Successional and Spatial Patterns of Bacterial Communities in Hydrocarbon-Contaminated Soils and *Populus* rhizosphere

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ACADEMIC DISSERTATION

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Summary

Ever-increasing urbanization and industrialization have led to contamination of a vast numbers of terrestrial sites with petroleum hydrocarbons. Petroleum hydrocarbon pollution has a deleterious impact on biotic and abiotic properties of ecosystem and can thereby affect some valuable ecosystem services. Microbes have the ability to metabolize various components of these harmful contaminants; this unique ability has been harnessed for decades in form of bioremediation and rhizoremediation, with varying success. Advanced knowledge on ecology of microbes in contaminated ecosystems can pave the way for improved design, optimization and monitoring of suitable bio-/rhizoremediation regimes in the clean-up of polluted sites. Recent developments in the molecular microbiology have enabled us studying microbial community structure, function and dynamics at great resolution and precision.

In this thesis, two important dimensions of microbial ecology in polluted ecosystems were explored: temporal and spatial. Succession of microbial communities in contaminated soil and *Populus* rhizosphere were studied in two different experiments. A short-term greenhouse study was conducted to monitor the immediate response of rhizosphere-associated and soil bacterial communities to oil pollution. We further scaled up our study to monitor the bacterial succession during a 2-year field study which also allowed us to analyse the effect of seasonal variation in boreal climate zone. Finally, a case study on an aged creosote-contaminated site located in South-eastern Finland was carried out in order to investigate the spatial patterns of microbial diversity and activity in relation to the heterogeneity of soil chemical parameters. Dynamics and diversity of microbial communities were accessed by employing T-RFLP fingerprinting and 454 pyrosequencing of structural and functional marker genes.

Successional changes in microbial communities could be observed in both our time-series experiments. High resolution sequencing of phylogenetic and catabolic marker genes for microbial community profiling not only enabled us to identify the bacterial groups during different stages of succession but also provided some insights on the structure-function relationship of bacterial communities. A gradual shift from specialist to generalist strategy was observed in the communities of aromatic and aliphatic degraders during the secondary succession in oil pollution. Effect of *Populus* rhizosphere on the general bacterial community structure was masked by the heavy oil pollution but upon careful examination of catabolic gene communities, rhizosphere-prevalent groups were observed. A significant variation in bacterial community structure was observed during the winter months pointing towards a distinct seasonal effect.

Our study on spatial heterogeneity of microbial communities in an aged contaminated site highlighted niche differentiation as the major mechanism regulating bacterial community structure. Geostatistical modelling and spatial prediction brought forward two distinct patterns in geochemical properties - patchy distribution of creosotes and a natural gradient of pH on the polluted site. While most bacterial taxa drastically reduced in abundance in the hotspots of pollution, Proteobacteria clearly dominated these zones. Acidobacteria, on the other hand, responded only to the pH variation irrespective of the differences in pollution levels. Analysing the behaviour of bacterial groups at lower taxonomic levels further clarified the patterns of

niche differentiation created by combined effect of pH and contaminants. The spatial profiles of specific microbial taxa could be used as proxies or indicators for monitoring this polluted site.

The results obtained in this thesis project are not only scientifically interesting but they also find an application in real-time ecological restoration. Both of our studies on bacterial secondary succession were carried out as a part of a phytoremediation project in collaboration with the Finnish Forest Research Institute. Phytoremediation with hybrid aspen is being implemented at the creosote-contaminated site in Luumäki, Finland (2013-). Our results on the spatial heterogeneity of microbial diversity and activity played a great role in the pre-evaluation of this site for remediation. This knowledge on the spatial patterns of microbial diversity will be highly useful in the coming years for the monitoring and evaluation of phytoremediation in the creosote-polluted site.

List of original articles

This thesis is based on the following publications:

I. **Mukherjee S**, Heinonen M, Dequivre M, Sipilä T, Pulkkinen P & Yrjälä K (2013). Secondary succession of bacterial communities and co-occurrence of phylotypes in oil-polluted *Populus* rhizosphere. *Soil Biology and Biochemistry* 58: 188–197.

II. **Mukherjee S**, Sipilä T, Pulkkinen P & Yrjälä K (2014). Secondary successional trajectories of structural and catabolic bacterial communities in oil-polluted soil planted with hybrid poplar. Submitted manuscript.

III. **Mukherjee S**, Juottonen H, Siivonen P, Quesada C-L, Tuomi P, Pulkkinen P & Yrjälä K (2014). Spatial patterns of microbial diversity and activity in an aged creosote-contaminated site. *The ISME Journal* doi: 10.1038/ismej.2014.151.

Authors' contributions

	I	**II**	**III**
Study design	**SM**, MH, TS, PP& KY	**SM**, PP, KY	**SM**, PT, PP, KY
Sampling	MD, MH, **SM**	**SM**	Golder associates**, SM**, PS
Microbial molecular analyses	**SM**, MD	**SM**	**SM**
Other analyses	**SM**, MH	**SM**	**SM**, PS, CLQ
Data handling	**SM**	**SM**, TS	**SM,** HJ
Writing the manuscript	**SM**, KY	**SM,** KY	**SM,** HJ

SM= Shinjini Mukherjee, MH= Mirja Heinonen, MD= Magali Dequivre, TS= Timo Sipilä, KY= Kim Yrjälä, PP= Pertti Pulkkinen, PT= Pirjo Tuomi, PS= Pauli Siivonen, CLQ= Cosme Lloret Quesada, HJ= Heli Juottonen

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AlkB	alkane monooxygenase
alkB	gene encoding alkane monooxygenase
AlmA	flavin-binding monooxygenase
ARISA	automated ribosomal intergenic spacer analysis
BLAST	Basic local alignment search tool
bp	base pairs
bphC	gene encoding 2,3-dihydroxybiphenyl-1,2-dioxygenase
BTEX	benzene, toluene, ethylbenzene and xylene
cDNA	complementary DNA
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDO	extradiol dioxygenase
EST	expressed sequence tag
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GST	glutathione S-transferase
gyrB	gene encoding the DNA gyrase subunit B
LadA	long-chain alkane hydroxylase
LH-PCR	length heterogeneity polymerase chain reaction
MPN	most probable number
MTBE	methyl terbutyl ether
NADH	reduced nicotinamide adenine dinucleotide
nahC	gene encoding 1,2-dihydroxynaphthalene-dioxygenase
NGS	next generation sequencing
NM-MDS	non-metric multidimensional scaling
NPMANOVA	non-parametric multivariate analysis of variance
OTU	operational taxonomic unit
PAH	poly aromatic hydrocarbon
PCA	principal component analysis
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
pMMO	particulate methane monooxygenase
RDA	redundancy analysis
RFLP	restriction fragment length polymorphism
rpoB	gene encoding the beta subunit of RNA polymerase
rpoD	gene encoding the sigma subunit of RNA polymerase
rRNA	ribosomal ribonucleic acid
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sMMO	soluble methane monooxygenase
SSCP	single-strand conformation polymorphism
TCE	trichloroethylene
TGGE	temperature gradient gel electrophoresis
TNT	trinitrotoluene
TPH	total petroleum hydrocarbon
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
VOC	volatile organic compound

1. Introduction

It has long been envisaged that polluted environments can be efficiently remediated through the application of amenable and controllable microbial activities. While this conjecture has been ably supported by some promising laboratory results, the application of pollutant-degrading microbes in field scenarios has mostly ended in disappointment. It has been proposed that for more optimal and rationale-driven remediation, it would be desirable to utilise the inherent catabolic potential of polluted sites. Currently however, there are limitations in harnessing this potential because of the lack of thorough understanding of ecological behaviour of microbial communities and their optimum catabolic capacity in such sites. It is noteworthy that, degradation of contaminants in polluted environments is mainly carried out by a microbial food web rather than a single species. In such situations, the key species and catabolic genes/enzymes involved are often different from those characterised in a laboratory setting. Recent advancements in molecular techniques have enabled us to determine the structural and catabolic microbial diversity at unprecedented resolutions. Harnessing these advancements in molecular microbial ecology would better guide our efforts towards sustainable remediation of polluted sites. In the following chapters, I explore some specific aspects of microbial ecology in hydrocarbon contaminated ecosystems.

1.1. Hydrocarbon contamination in terrestrial ecosystems

Petroleum products are fundamental to our lives in form of transportation fuels, heating and power-generating fuels and many other useful derivatives. The volume of petroleum products used today dwarfs all other chemicals of environmental health and concern. Extraction, refinement and transportation of petroleum have resulted in surface and near-subsurface soil contamination with crude oil, gasoline, diesel and creosote. The composition of released petroleum products varies significantly depending on the source, the physical, chemical and biological changes (collectively referred to as weathering) of the product over time, and differential movement of the components in the environment. Crude oil is a very complex mixture of more than several thousand distinct chemical components which can be further classified into following main groups of chemicals: aliphatics including alkanes, alkenes and cycloalkanes, aromatics which include monoaromatic and polyaromatic hydrocarbons, asphaltenes, resins and traces of metals (Petrov, 1987; Mullins et al, 2007; Marshall & Rodgers, 2004) . Creosote, a by-product of tar distillation, is composed approximately of 85% polycyclic aromatic hydrocarbons (PAHs), 10% phenolic compounds; and 5% N-, S-, and O-heterocyclics (Mueller et al, 1989). Among these groups of chemicals, PAHs are of foremost environmental concern due to their recalcitrance (Bamforth & Singleton, 2005; Cerniglia, 1993) and toxicity (Douben, 2003; Bispo et al, 1999; Harvey, 1996) . Monoaromatics, such as benzene, toluene, ethylbenzene, and xylenes (BTEX), are also some of the most common pollutants which are frequently encountered on contaminated sites. Similarly, alkanes pose a significant problem due to their inertness and viscosity (Head et al, 2006). Chain length of alkanes is the major determinant of their bioavailability and toxicity. Short chain n-alkanes act

as solvents for cellular membranes and fatty compounds and are directly toxic whereas, long chain n-alkanes have deleterious effect on the macro- and microflora by forming oil films.

1.2. Hydrocarbons as a food source for microbes

About a century ago, the ability of bacterial isolates to use aliphatic and aromatic hydrocarbons as sole carbon and energy sources was reported by Söhngen (1913) . Since then, many phyla of bacteria such as Proteobacteria (α-, β- and γ-), Actinobacteria, Firmicutes, Deinococcus-Thermus and Bacteriodetes, filamentous fungi and yeasts have been found to be involved in alkane degradation. In addition to being an important component of crude oil (20-50%), alkanes are also produced by plants, algae and bacteria (Hornafius et al. 1999; Seewald 2003 Widdel and Rabus 2001); that explains the presence of small amounts of alkanes in the pristine ecosystems. It has been observed that pristine ecosystems- both terrestrial and aquatic, contain some amount of hydrocarbon-degrading microorganisms, and their relative abundance is considerably increased in oil-polluted sites. Alkane degraders usually have a versatile metabolism and they can utilize a vast array of carbon sources in addition to alkanes. Most alkane degraders are facultative and they prefer simpler carbon sources before turning to alkanes but some obligate alkane degraders, also known as hydrocarbonoclastic bacteria, have also been reported from polluted environments. *Alcanivorax*, *Thalassolituus*, *Oleivorans* (Yakimov et al. 1998, 2004) and *Oleispira* are some genera of hydrocabonoclastic bacteria found to be involved in biodegradation of oil spills in several environments (McKew et al., 2007; Coulon et al., 2007).

PAHs are introduced in the environment via pyrolysis of biomass. It is well known that living organisms produce certain benzenoid compounds (Moshier and Chapman, 1973). Moreover, plants produce a vast array of aromatic compounds such as flavonoids, chromenes, dibenzofurans, cresols, xanthones, and lignin. The potential to degrade aromatic compounds is widespread in bacteria, and in the natural environment these bacteria contribute to the breakdown of aromatic compounds and to the global carbon cycle. Due to the aromatic ring structure, degradation pathways for different PAHs are much more diverse and complex compared to alkanes. While some microbes can completely mineralize few PAH compounds, most individual species do not harbour all the enzymes required for the whole pathway of degradation. In most scenarios, many different microbial groups with diverse enzyme systems are involved in the degradation of petroleum hydrocarbons. Elaborate knowledge of microbial populations capable of thriving on specific components of petroleum under various environmental conditions would therefore be highly desirable.

1.3. Rhizoremediation of petroleum hydrocarbon polluted soils

Owing to the catabolic potential and versatility of microorganisms (mainly bacteria and fungi), bioremediation is an effective way of cleaning up petroleum hydrocarbon contaminated soil. Success of a bioremediation regime depends, however, on several factors which might govern the colonization, niche preferences, survival, growth and reproduction of suitable microbes in contaminated soils. These factors include nutrient availability, temperature, pH, oxygen, moisture, toxicity and bioavailability of contaminants to name a few (Boopathy, 2000; Vidali,

2001). By gaining an understanding and accordingly manipulating these factors, effectiveness of bioremediation can vastly be improved. Phytoremediation, *i.e.* use of plants for remediation has been proposed and applied as an alternative strategy for the removal of organic pollutants (Macek et al, 2000; Susarla et al, 2002; Schnoor et al, 1995; White & Newman, 2011). Depending on the mechanism by which remediation of organics can be achieved, common phytoremediation strategies include:

- Phytovolatilization: plants take-up the contaminant from soil and after transforming it into a volatile compound, release it into the atmosphere (Arnold et al, 2007; Doucette et al, 2003).
- Phytoaccumulation/phytoextraction: organics or metals are removed by pollution-accumulating plants as the contaminants are concentrated in the harvestable parts of plants (Boonsaner et al, 2011).
- Phytodegradation/phytotransformation: enzymes derived from plants and/or their associated microbes degrade organic contaminants (Dec & Bollag, 1994; Newman et al, 1997).
- **Rhizoremediation**: Enhanced degradation of organics by microbes living in the rhizosphere of plants (Kuiper et al, 2004; Gerhardt et al, 2009; Leigh et al, 2007; Mackova et al, 2009).

Some of these mechanisms may also function simultaneously during the remediation of contaminants by plants.

Rhizoremediation has been shown to be an effective strategy for the clean-up of petroleum hydrocarbon contaminated soils (Ramos et al, 2010; Mackova et al, 2009; Mikkonen et al, 2011; Hong et al, 2011; Yateem et al, 2007). The importance of association of plant roots with soil microorganisms has been recognized since the early 20th century when Hiltner (1904) defined "rhizosphere" as the zone of soil where microbes are influenced by the root system. Apart from the physical influence of roots (enhanced aeration in soil, microbial attachment site, nutrient transport etc.), enhanced degradation of petroleum hydrocarbons in the rhizosphere can mostly be attributed to the root exudates. Root exudates can be generally categorized as organic polymers (from root debris and sloughing cells), low molecular weight carbohydrates, amino acids, organic acid anions and an array of secondary metabolites (Walker et al, 2003; Bais et al, 2006). Fig. 1 summarizes some of the important mechanisms by which root exudates can enhance the degradation of petroleum hydrocarbons in rhizosphere. These mechanisms are briefly described as follows (Martin et al, 2014):

A) Direct degradation: plant roots may secrete enzymes such as laccases and peroxidases which may catalyse the oxidation of some PAHs common in fuel mixtures (Gerhardt et al, 2009).

B) Co-metabolism, enzyme induction/selective enrichment: Many secondary metabolites in root exudates structurally resemble PAHs common in petroleum hydrocarbons. Hence, co-metabolism of PAHs along with the plant secondary metabolites is a major route of degradation of recalcitrant hydrocarbons (Cunningham et al, 1995; Fletcher & Hegde, 1995).

C) Increased bioavailability: It is well known that microorganisms can secrete biosurfactants which increase the solubility of certain organics and also favour the attachment

of microbes to oil droplets. Similarly, root exudates may also contain lipophilic compounds which promote the bioavailability of petroleum hydrocarbon compounds (Read et al, 2003).

D) Degradation via mycorrhizal symbionts: Extracellular enzymes secreted by mycorrhizal symbionts of plants can degrade some aliphatic and aromatic components of PHCs (Harms et al, 2011).

E) Increased biomass and/or activity- This is perhaps the most commonly mentioned role of root exudates, also referred to as the rhizosphere effect. Increased supply of nutrients and energy to microbes via root exudates leads to a higher abundance and activity of microbes in the rhizosphere.

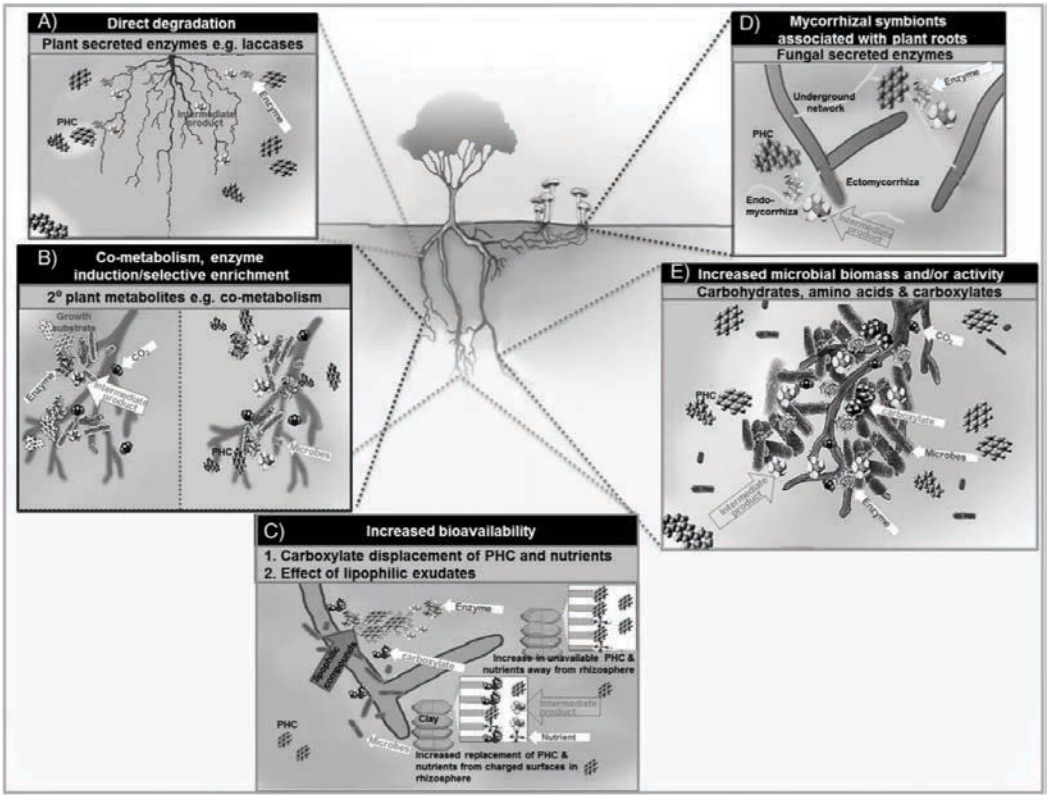


Fig. 1: Mechanisms by which root exudates can enhance biodegradation of petroleum hydrocarbons. Source: Martin et al., 2014

1.4. Poplars (aspen) for remediation of organics

Trees and perennial grasses are preferentially employed in phytoremediation as unlike annual plants, they do not need yearly replantation. Easy propagation, fast growth, phreatophytic root systems, high water uptake rate, high absorption surface areas and tolerance to contaminants are some of the important properties that make some tree species a practical choice for phytoremediation (Cook & Hesterberg, 2013). Among the most frequently studied tree species in context of phytoremediation of organic contaminants are poplars (Populus spp.), willows

(Salix spp.), red mulberry (Morus rubra) and birches (Betula spp.)(Vervaeke et al, 2003; de Carcer et al, 2007; Yrjälä et al, 2010; Sipilä et al, 2008; Tervahauta et al, 2009).

Populus is a member of the Salicaceae family, which also includes willows. In addition to around 30 species occurring in the northern hemisphere, *Populus spp*. also have the ability to cross within the genus, both in the wild and through controlled breeding, giving rise to a number of potential clones (Dickmann and Stuart 1983). Due to their ability to readily form hybrids, poplars have been crossed by foresters for years in order to maximize growth rates and yield (Klopfenstein et al, 1997). *Populus* has been considered a model woody plant due to its relatively modest genome size, rapid early growth, ease of clonal propagation, and routine transformation protocols (Bradshaw et al, 2000; Taylor, 2002; Wullschleger et al, 2002). Sequencing of the whole genome of *Populus trichocarpa* and availability of an extensive expressed sequence tag (EST) database has further added to the long list of importance research attributes of *Populus* species (Sterky et al, 2004; Tuskan et al, 2006). Owing to these advantages, hybrid poplars were originally bred and used as cash crops for pulp and energy. In past two decades, hybrid and transgenic poplars have extensively been utilized in the remediation of a broad range of organic contaminants (Table 1). It can be comprehended that poplars can remediate organic contaminants via various mechanisms including phytovolatilization, phytodegradation as well as rhizoremediation. This literature survey clearly indicates a knowledge gap regarding the structural and catabolic bacterial communities in polluted- Populus rhizosphere. Dynamics of rhizosphere associated bacterial communities in hybrid aspen (*Populus tremula* × *Populus tremuloides*) growing in hydrocarbon contaminated soils have been explored in this thesis project (**I &II**).

Table 1: Summary of studies involving poplars for remediation of organic contaminants

***Populus* species**	**Organic contaminant**	**Theme of study**	**Reference**
Populus deltoids × *nigra* DN34	Atrazine	Vegetative uptake and degradation of atrazine in the rhizosphere.	Burken & Schnoor, 1996
Populus deltoides × *nigra* DN-34, Imperial Carolina	Atrazine & benzene, toluene, and xylenes (BTX)	First report of enumeration of specific microbial populations (total heterotrophs, denitrifiers, pseudomonads, BTX degraders, and atrazine degraders) in *Populus* rhizosphere.	Jordahl et al, 1997
Populus trichocarpa × *P. deltoids*	Trichloroethylene (TCE)	A comprehensive study demonstrating efficient metabolism of TCE by axenic cultures of Populus cells, poplar cuttings and in a field trial with poplar trees.	Gordon et al, 1998
Populus deltoids × *nigra* DN-34	Benzene	Laboratory experiments investigating the toxicity response of poplar cuttings to benzene exposure, contaminant distribution in plant tissues, contaminant degradation in the soil profile, and contaminant volatilization from the soil and plant tissues.	Burken et al, 2001
Populus deltoides × *nigra* DN-34, Imperial Carolina	Methyl tert-butyl ether (MTBE)	A 3 phase study involving- A) a laboratory bioreactor study that examined the fate and transport of ^{14}C-radiolabeled MTBE in hybrid poplars B) mathematical modelling investigating the influence of deep-rooted trees on unsaturated and saturated groundwater flow & C) a field study at a site with MTBE-contaminated groundwater where hybrid poplar trees were planted.	Hong et al, 2001
Populus nigra	Diesel	A report on effect of diesel fuel on plant growth and on the rhizosphere microflora. T-RFLP fingerprinting of rhizosphere bacterial communities & isolation and identification of hydrocarbon-degrading strains was performed and these strains were checked for the presence of *alkB* gene.	Tesar et al, 2002
Populus deltoides × *Populus trichocarpa*	Volatile organic compounds (VOCs)	Phytoremediation field experiment demonstrating that hybrid poplar trees mitigate the migration of a groundwater plume of volatile organic compounds.	Hirsh et al, 2003
Populus deltoides × *nigra* DN-34	RDX	Poplar tissue cultures and leaf crude extracts were shown to mineralize RDX upon exposure to light.	Van Aken et al, 2004
P. trichocarpa × *deltoides* cv. Hoogvorst	Toluene	First report of *in planta* horizontal gene transfer among plant-associated endophytic bacteria. Inoculation of *Burkholderia cepacia* VM1468 containing pTOM-Bu61 plasmid (carrying genes for toluene degradation) had a positive effect on plant growth in the presence of toluene.	Taghavi et al, 2005

Populus trichocarpa × *deltoids* cv. "Hazendans" and "Hoogvorst"	BTEX	Endophytic bacteria were isolated from the root, stem and leaf of two cultivars of poplar tree growing on a site contaminated with BTEX compounds. They were further characterised genotypically (16S rRNA sequencing) and phenotypically by their tolerance to target pollutants. The endophytic bacteria exhibited marked spatial compartmentalisation within the plant.	Moore et al, 2006
Populus deltoides × *nigra* DN-34	RDX	First report showing that the exposure of poplar plants to RDX results in the induction of several genes that are involved in explosive detoxification such as glutathione S-transferases (GSTs), cytochrome P-450s (CYPs), NADPH-dependent reductases, and peroxidases.	Tanaka et al, 2007
Populus deltoides × nigra, DN34	Polychlorinate-d biphenyls (PCB)	Report on the transport of PCBs through whole plants designed for use in treatment at disposal facilities	Liu & Schnoor, 2008
Transgenic *Populus tremula* × *tremuloides* var. Etropole	2,4,6-trinitrotoluene (TNT)	Transgenic hybrid poplars expressing bacterial nitroreductase gene, *pnrA*, were shown to tolerate and take-up greater amounts of TNT	Van Dillewijn et al, 2008
Populus deltoids × (*Populus trichocarpa* × *Populus deltoides*) cv.Grimminge	TCE	First in situ inoculation of poplar trees, growing on a TCE-contaminated site, with the TCE-degrading strain Pseudomonas putida W619-TCE. P. putida W619-TCE was extablished and enriched *in planta* as a poplar root endophyte and by further horizontal gene transfer of TCE metabolic activity to members of the poplar's endogenous endophytic population.	Weyens et al, 2009
Populus tremula × *P. alba* and *P. trichocarpa*	Chlorpyrifos	Hydroponic study demonstrating the efficiency of poplars in uptake and translocation of Chloropyrifos.	Lee et al, 2012
Populus nigra (var. italica)	Petroleum hydrocarbons, PCBs and metals	Phytoremediation field study at a site historically contaminated with petroleum hydrocarbons, PCBs and metals; poplars together with horse manure treatment were shown to be effective in remediation. Biogeochemical parameters were monitored by SDS PAGE.	Doni et al, 2012
Populus deltoides × *Populus nigra*	Poly aromatic hydrocarbons (PAHs)	Efficacy of *Burkholderia fungorum* DBT1 (a strain isolated from oil refinery discharge and capable of degrading dibenzothiophene, phenanthrene, naphthalene, and fluorene) was demonstated as an endophyte in poplars during 18 weeks greenhouse experiment	Andreolli et al, 2013

1.5. Monitoring microbial populations in contaminated ecosystems using culture-independent tools

The "great plate count anomaly" implies that the readily cultured microbes are like the 'weeds' of microbial world and they represent less than 1% of the total microbial diversity (Staley & Konopka, 1985). This figure (1%) was however estimated by comparing plate counts with direct microscopic counts of microorganisms in environmental samples and has been criticized (Donachie et al, 2007). The pure culture based studies still retain their significance for the development and interpretation of molecular analyses in microbial ecology. Nevertheless, the tremendous amount of information generated by the application of molecular techniques has transformed our view of the microbial world (Hugenholtz, 2002). The key idea behind this approach was to explore the uncultured microbial diversity by retrieving and sequencing the macromolecules directly from environmental samples (Pace, 1997). While, the initial progress with this approach was mostly focused on the phylogenetic marker - 16S rRNA gene, techniques targeting specific functional marker genes and metagenomes have been developed and used over the recent decade (Handelsman, 2004; Albertsen et al, 2013; Van Elsas & Boersma, 2011; Hirsch et al, 2010). The inherent complexity of microbial communities has become quite evident. Culture-independent techniques are based on a holistic approach i.e. studying a natural habitat directly as opposed to a reductionist approach, i.e. studying each organism in isolation.

1.5.1. DNA based microbial community profiling

DNA based community profiling generally begins with the direct isolation of DNA from environmental samples followed by polymerase chain reaction (PCR) amplification of marker genes using universal primers capable of amplifying the target genes from a broad range of taxa (Nocker et al, 2007). The marker gene used could either be a phylogenetic marker like 16S rRNA gene or a functional gene encoding for a specific protein. The PCR products (amplicons) are further subjected to a suitable profiling method (Torsvik & Ovreas, 2002; Muyzer & Smalla, 1998; Liu et al, 1997; Suzuki et al, 1998; Fisher & Triplett, 1999; Dohrmann et al, 2004; Schwieger & Tebbe, 1998). Most profiling methods achieve the differentiation in the PCR products based on:

A) Location of restriction endonuclease sites: Terminal restriction fragment length polymorphism (T-RFLP), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA, specific for 16S rRNA genes)

B) Melting behaviour of double stranded PCR products related to the sequence composition and primary structure of target gene fragments: Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE)

C) Electrophoretic mobility of single stranded DNA fragments in non-denaturing gels: Single-strand conformational polymorphism (SSCP)

D) Variation in length of entire gene fragments: Length heterogeneity PCR (LH-PCR), automated ribosomal intergenic spacer analysis (ARISA).

The resolution of these profiling methods varies considerably and selection of a suitable method is important for the accurate interpretation of microbial community structure and dynamics.

An alternative approach is amplicon sequencing of target genes and comparing the sequence composition *in silico* for assessing the differences in community structure. Clone library sequencing has been the traditional method of choice for a long time and has helped us to describe a considerable fraction of structural and functional microbial diversity. Nonetheless, even the most extensive clone library based sequencings have underestimated microbial diversity due to under-sampling (Curtis & Sloan, 2005; Quince et al, 2008). In the last few years, next generation sequencing (NGS) platforms such as 454 pyrosequencing, Illumina, SOLiD, Ion Torrent, Ion proton, PacBio and Oxford Nanopore technologies have revolutionized the field of sequencing by the massive amount of data produced at reduced price and time. Due to the possibility of 'barcode –tagging' which enables massive screening of multiple samples, some of these next generation sequencing methods are becoming a potential way of microbial community profiling.

In this thesis, **T-RFLP (I) and 454 amplicon sequencing (II &III)** have been employed for the profiling of structural and functional microbial communities. Some technical aspects related to these two methods are discussed in the following paragraphs.

Terminal restriction fragment length polymorphism

T-RFLP is one of the most widely used fingerprinting methods in microbial ecology owing to its relative simplicity, great reproducibility and provision for robust *in silico* data analysis (Liu et al, 1997; Osborn et al, 2000; Schütte et al, 2008; Abdo et al, 2006; Dollhopf et al, 2001). It has frequently been used not only for analysing 16S rRNA genes (Sipilä et al, 2008; Mummey & Stahl, 2003; Yrjälä et al, 2010) but also functional genes; e.g. genes involved in hydrocarbon degradation such as alkane hydroxylases (Giebler et al, 2013; Schulz et al, 2012; Paisse et al, 2011), ring hydroxylating dioxygenases (Vitte et al, 2013) genes involved in nitrogen metabolism (Bannert et al, 2011; Wessén et al, 2011) and methane production (Juottonen et al, 2012; Yrjälä et al, 2011).

Briefly, the gene of interest is amplified using fluorescently labelled primers (either one or both the primers can be labelled); the resulting labelled amplicons are then digested with one or more restriction enzymes and the final step involves the determination of size and relative abundances of the fluorescently labelled terminal restriction fragment (T-RF) using an automated DNA sequencer. The differences in the length (size in base pairs) of T-RFs depict the difference in sequence composition of the target gene and thus ideally represent distinct microbial groups. It is also possible to identify specific T-RFs by cloning and sequencing the target gene from the same environmental sample. With a suitable data-analysis approach, T-RFLP can be applied to assess treatment- specific, temporal or spatial changes in microbial communities. One should bear in mind while designing or using T-RFLP protocols that this method shares the technical limitations associated with PCR; for instance, selection of appropriate primer pairs can be tricky as they should be specific but still have a broad coverage of multiple taxa. Additionally, partial digestion of amplicons can lead to a faulty representation of diversity.

454 pyrosequencing

The 454 pyrosequencing platform is a pioneering landmark in the field of next-generation sequencing because it advanced the field in unprecedented ways beyond three main bottlenecks i.e. library preparation, template preparation and sequencing (Margulies et al, 2005; Ronaghi et al, 1998; Ronaghi et al, 1996). The impact of 454 can be gauged on the basis of the fact that, the solutions it provided have since been and still continue to be emulated by successive next-generation sequencing platforms (Shendure & Ji, 2008; Rothberg & Leamon, 2008; Metzker, 2009). Since its arrival, 454 pyrosequencing has become a popular choice for studying the microbial diversity in diverse environments through 16S rRNA amplicon sequencing (Sogin et al, 2006; Roesch et al, 2007; Will et al, 2010; Leininger et al, 2006; Lauber et al, 2009), for metagenomic (Dinsdale et al, 2008; Edwards et al, 2006; Mackelprang et al, 2011) and metatranscriptomic (Poretsky et al, 2009; Gifford et al, 2010; Lehembre et al, 2013) studies as well as microbial genome sequencing (Paliwal et al, 2014; Goodison et al, 2013). Application of 454 sequencing has also added to our understanding of microbial community structure and dynamics in polluted ecosystems (Uhlik et al, 2012; Kotik et al, 2013; Dos Santos et al, 2011; Singleton et al, 2011).

An overview of 454 pyrosequencing workflow and summary of steps involved are presented in Fig. 2. The raw data generated from 454 pyrosequencing is a series of images which are normalized and converted into flowgrams (SFF files). SFF files are the starting point of sequence data analysis. One can either begin the pre-processing of 454 sequences with the flowgrams or use the Fasta files and proceed with the standard bioinformatics tools. Flow values in the SFF files, however, can provide additional information about sequence quality that is not available in the pure nucleotide sequences. Next step after pre-processing and denoising is usually alignment of sequences (unsupervised or alignment to reference databases); alignment is one of the most important steps in the process of quality screening of sequences. Next, removal of chimeric sequences is performed. Good quality sequences are then clustered into operational taxonomic units (OTUs) and microbial diversity and community structure are further analysed via downstream statistical analysis.

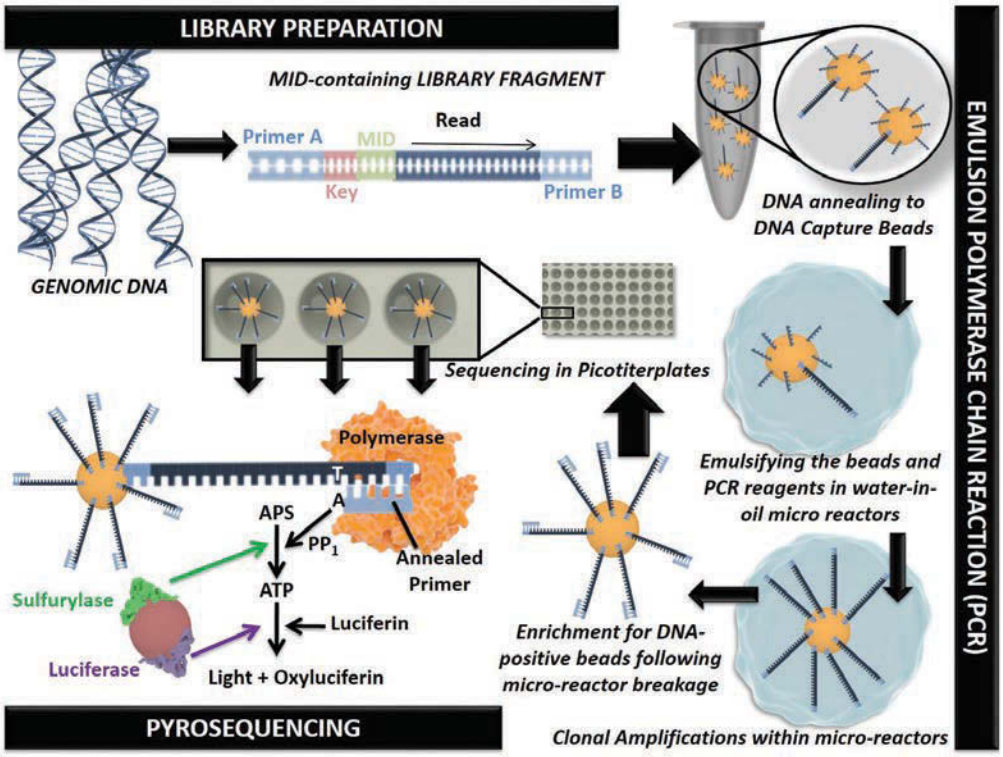


Fig. 2: A schematic illustration of the different steps in 454-sequencing. A) a library of amplicons/fragmented DNA or cDNA ligated to specific 5′- and 3′-end specific adapters is prepared B) followed by emulsion PCR wherein, each emulsion droplet behaves as a microreactor for PCR amplification, C) Beads with amplified fragments bound to them are released from emulsion and are loaded onto a picotiter plate with the capacity of one bead per well. Pyrosequencing takes place in the picotiter plates by a sequential flow of sequencing reagents across the plate, when a complementary nucleotide is added to a particular template in an extension reaction and a light signal is generated giving rise to flowgrams.(modified from Rothberg et.al. 2008)

1.5.2. Phylogenetic and functional/catabolic marker genes

Use of gene sequences as molecular clocks to decode phylogenetic relationships was first proposed by Pauling and Zuckerkandl (1965). The landmark study of Carl Woese and colleagues on the small subunit ribosomal RNA (16S rRNA and 18S rRNA) provided a framework for determining phylogenetic relationships among organisms (Woese, 1987; Woese et al, 1975). This pioneering work altered our view of evolution from a five kingdom to a three domain (Eukaryota, Bacteria and Archaea) paradigm (Woese & Fox, 1977; Woese, 1987; Woese et al, 1990). 16S rRNA gene has been established as the "gold standard" in microbial ecology studies and is by far the most commonly used phylogenetic marker due to its properties such as ubiquity, extreme sequence conservation, a domain structure with variable evolutionary rates and highly developed databases (Tringe & Hugenholtz, 2008). Nonetheless, existence of

multiple heterogeneous copies of the 16S rRNA gene within a single genome can hamper robust species differentiation (Crosby & Criddle, 2003). Alternative phylogenetic markers such as *rpoB*, *rpoD*, *recA* and *gyrB* have also been used (Case et al, 2007; Wang et al, 2007; Thompson et al, 2004). While 16S rRNA gene based community profiling gives a view of microbial community composition, it hardly can provide information on the community functions. This is due to the fact that phylogenetically unrelated groups of organisms can often carry out similar ecological processes and structural shifts in community does not always relate to corresponding functional differences. For this reason, genes coding for enzymes, which carry out specific metabolic processes, can give a better description of functional aspects of communities. In contaminated ecosystems, genes for catabolic enzymes involved in biodegradation pathways can be useful as functional markers for tracking the microbial groups capable of feeding on specific pollutants. Comparative and integrated analysis of phylogenetic and functional markers can possibly provide a better link between community structure and function.

1.5.3. Extradiol dioxygenase as a marker for aromatic degraders

As discussed in previous sections, PAHs are prominent environmental pollutants and therefore the enzymes involved in aromatic degradation pathways are of considerable interest. The aromatic ring system in these compounds makes them inert as substrates towards simple oxidation or reduction and thus requires elaborate degradation strategies. Given the vast variety in structure of aromatic compounds, it would take as many metabolic pathways as there are compounds for the microbes to make use of all of them if there was no coordination in their degradation. Most of the aromatic compounds are, however, channeled by **upper /peripheral pathways** into **lower/ central pathways** by their transformation to a few key central intermediates. Central/lower pathways then convert these intermediates into metabolites such as acetyl-CoA, succinyl-CoA and pyruvate (Fuchs et al, 2011). Aerobic and anaerobic environmental conditions further regulate the strategies of PAH degradation. The majority of reported bacterial aromatic degradation processes are aerobic but alternative strategies under low O_2 levels or even O_2-free conditions have also been identified (Gibson & S. Harwood, 2002; Heider & Fuchs, 1997; Meckenstock et al, 2004) .

Under aerobic conditions**, ring hydroxylating diooxygenases** oxidize aromatic rings by addition of both atoms of molecular oxygen to two adjacent carbon atoms to produce cis-dihydrodiols which are further acted upon by **cis-dihydrodiol dehydrogenases.** In the next step, **ring-cleavage dioxygenases** catalyze a reaction in which molecular oxygen is inserted into a C–C bond of dihydroxylated aromatic compounds, resulting in cleavage of the ring (Fig. 3). Two classes of **ring-cleavage dioxygenases** have been identified, on the basis of the mode of ring cleavage: **Intradiol dioxygenases** utilize non-haeme Fe III to cleave the aromatic nucleus *ortho* to (that is, between) the hydroxyl substituents, whereas **extradiol dioxygenases (EDO)** utilize non-haeme Fe II or other divalent metal ions to cleave the aromatic nucleus *meta* to (that is, next to one of) the hydroxyl substituents (Vaillancourt et al, 2006; Lipscomb, 2008). In catabolism of polyaromatic hydrocarbons (PAHs), the first aromatic ring structure is cleaved by the upper *meta*-pathway EDOs and the second ring by lower *meta*-pathway EDOs (Williams and Sayers 1994, van der Meer 1997, Lloyd-Jones et al. 1999).

In this thesis project, genes coding for upper *meta*-pathway **I.3.E group EDOs (**I.3.E designates type (I), family (3) and subfamily (E)**)** have been targeted as a marker to capture the functional diversity related to polyaromatic degradation in Bacteria. The target subfamily I.3.E contains enzymes for initial ring-cleavage of biphenyl, naphthalene and evidently also for polyaromates such as dibenzothiophene and phenanthrene, containing three aromatic rings (Denome et al., 1993; Pinyakong et al., 2003). The primer pair employed for amplification of extradiol dioxygenases in articles **I & II** was designed by Sipilä et al (2006). This primer pair theoretically amplifies a 469-bp fragment of the *nahC* gene of *Pseudomonas putida* Nah7 plasmid at position 131 to 600bp (Sipilä et al, 2006). The efficacy of these primers in successful amplification of extradiol dioxygenases from pristine and contaminated soils and PAH polluted birch rhizospheres has previously been demonstrated (Yrjälä et al, 2010b; Sipilä et al, 2006; Sipilä et al, 2008) . Broad specificity of these primers, *i.e.*, efficient amplification from Alpha-, Beta-, Gammaproteobacteria and gram positives such as *Rhodococcus* further makes them ideal for monitoring aromatic degraders in bioremediation and rhizoremediation studies.

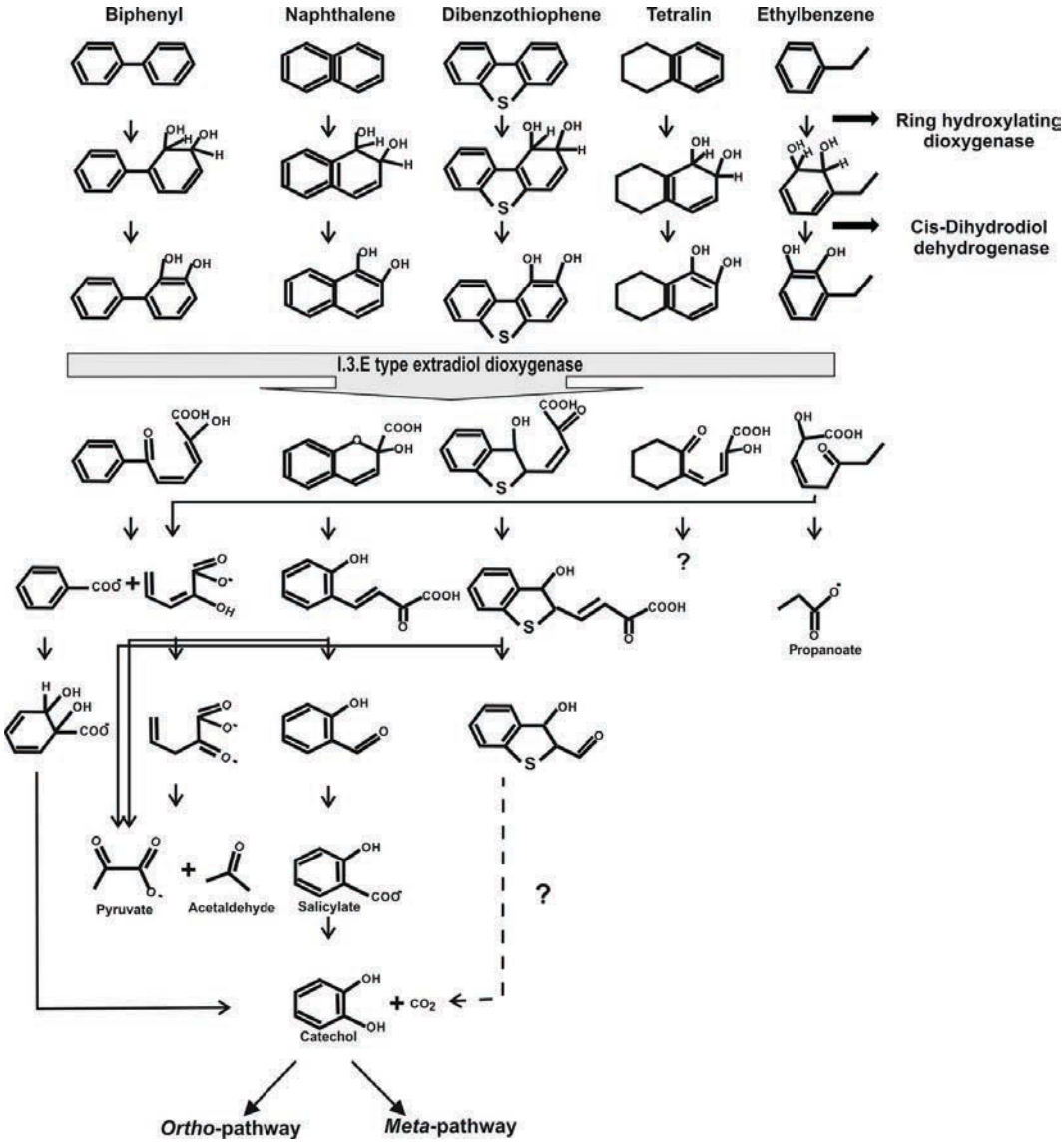


Fig. 3: Peripheral/ upper meta- pathway of degradation of aromatic compounds (biphenyl, naphthalene, dibenzothiophene, tetralin and ethylbenzene). 1.3 E type extradiol dioxygenase enzymes catalyze the third step in the degradation leading to cleavage of aromatic rings of these compounds. Adapted from Sipilä et al. 2009.

1.5.4. Alkane monooxygenase as a marker for aliphatic degraders

The ability to degrade alkanes under aerobic and anaerobic conditions is widespread among many phyla of Bacteria, some fungi and yeasts found in both pristine and polluted ecosystems (Rojo, 2009; Leahy & Colwell, 1990). Under aerobic conditions, different enzymes systems

initiate the biodegradation process by introducing oxygen in the alkanes based on their chain length (van Beilen & Funhoff, 2007). Different classes of these enzymes based on substrate range (alkane chain-length) have been listed in Table 2. It is noteworthy that the substrate ranges for these enzymes may overlap.

Among these classes of enzymes, the terminal oxidation pathway of alkanes involving **AlkB related alkane monooxygenases** has been most thoroughly studied (Rehm & Reiff, 1981; van Beilen et al, 2003; van Beilen & Funhoff, 2007). Fig. 4 represents the terminal pathway of alkane degradation and role of alkane monooxygenase as the key enzyme in the catabolism of medium chain length (C5- C16) alkanes. The functional Alk enzyme system comprises the transmembrane alkane monooxygenase AlkB (encoded by the *alkB* gene) and two co-factors named rubredoxin (AlkF) and rubredoxin reductase (AlkG). These co-factors are responsible for transferring the electrons involved in alkane hydroxylation by AlkB (van Beilen et al, 2001; van Beilen et al, 2003).The gene encoding for alkane monooxygenases (*alkB*) has been used as functional marker for tracking the abundance and diversity of alkane degrading bacteria in a variety of pristine (Schulz et al, 2012) and polluted soils (Bell et al, 2011; Pérez-de-Mora et al, 2011; Schloter, 2014; Whyte et al, 2002) and rhizosphere (Andria et al, 2009) . Primer pairs designed by Kloos et al (2006) were used for targeting the alkane degraders from oil polluted soil and rhizospheres in article **II** of this thesis. These primers were previously shown to successfully amplify *alkB* genes from Alpha-, Beta-, Gammaproteobacteria, Flavobacteria, Firmicutes and Bacilli from variety of soils (Jurelevicius et al, 2013).

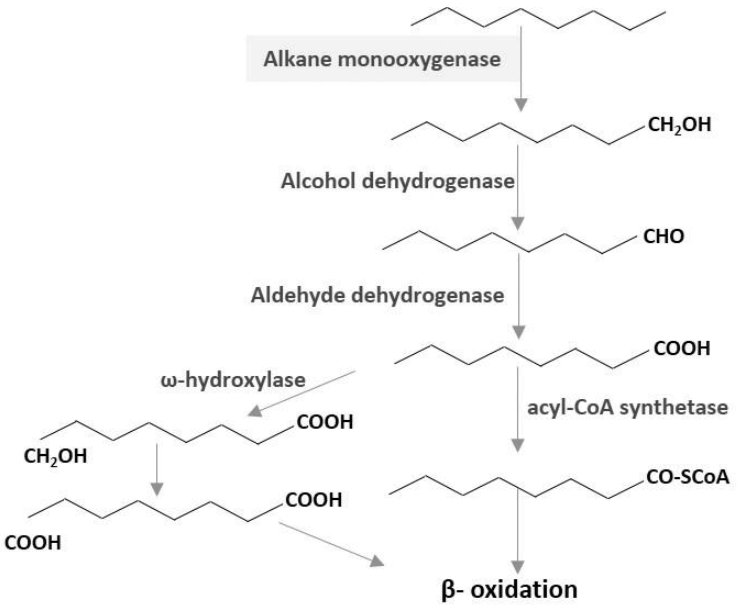


Fig. 4: Terminal oxidation pathway of alkane degradation. Alkane monooxygenase gene/enzyme studied in this thesis has been highlighted. Modified from Rojo, 2009

Table 2: Enzyme classes involved in aerobic degradation of alkanes

Substrate range	Enzyme class	Composition and cofactors	References
C1-C8 (halogenated)-alkanes, alkanes, alkenes, cycloalkanes	Soluble methane monooxygenase (sMMO)	α2β2γ2 hydroxylase; dinuclear iron reductase, [2Fe–2S], FAD, NADH regulatory subunit	Fox et al, 1990; Lipscomb, 1994; Oldenhuis et al, 1989; Vorobev et al, 2011
C1–C5 (halogenated)-alkanes, alkenes	Particulate methane monooxygenase (pMMO)	α3β3γ3 hydroxylase trimer composed of PmoA, PmoB, PmoC; mononuclear copper and dinuclear copper in PmoB	Rosenzweig, 2011; McDonald & Murrell, 1997; Semrau et al, 1995
C5–C16 alkanes, fatty acids, alkylbenzenes, cycloalkanes, etc	AlkB-related alkane monooxygenases	Membrane hydroxylase; dinuclear iron rubredoxin; mononuclear iron rubredoxin reductase, FAD, NADH	van Beilen & Funhoff, 2007; van Beilen et al, 2003
C5–C16 alkanes, (cyclo)-alkanes, alkylbenzenes, etc	Bacterial P450 oxygenase systems (CYP153, class I)	P450 oxygenase; P450 heme ferredoxin; iron–sulfur ferredoxin reductase, FAD, NADH	Funhoff et al, 2006; Bell et al, 2006; van Beilen et al, 2006; Bell et al, 2010
C10–C16 alkanes, fatty acids	Eukaryotic P450 (CYP52, Class II)	Microsomal oxygenase; P450 heme reductase; FAD, FMN, NADPH	Črešnar & Petrič, 2011; Yadav & Loper, 1999; Iida et al, 2000
C10 –C30 alkanes	Dioxygenase	Homodimer; copper, FAD	Maeng et al, 1996; Throne-Holst et al, 2007
C20 –C32 alkanes	AlmA type *n*-alkane monooxygenase	flavin-binding monooxygenase	Wang & Shao, 2012; Wentzel et al, 2007
C15 –C36	LadA type long-chain *n*-alkane monooxygenase	flavoprotein monooxygenase; NAD(P)H-dependent flavin reductase and monooxygenase	Li et al, 2008; Feng et al, 2007

1.6. Successional dynamics of microbes in polluted soils and rhizospheres

Comprehending the patterns of temporal changes in communities is one of the major pursuits in ecology. Ecological succession refers to a process of more or less deterministic developments in the composition or structure of an ecological community (Connell & Slatyer, 1977; Walker & Del Moral, 2003; Horn, 1974). In plant ecology, succession has typically been divided into two major categories: primary succession, which occurs on an un-colonized substrate (e.g. lava fields or glacial retreats) and secondary succession, which is triggered by a radical disturbance in a previously colonized environment (e.g. harvested agricultural fields or forest fire). Majority of our knowledge on the concepts and mechanisms of succession are derived from the field of plant ecology and microbial succession has received much less attention (Fierer et al, 2010). Recent advancements in culture-independent tools have helped microbial ecologists to overcome the methodological limitations in analysing the temporal variation of highly diverse and rapidly changing microbial communities in various ecosystems (Kowalchuk et al, 2000; Banning et al, 2011; Koenig et al, 2011; Wertz et al, 2007; Podell et al, 2014). Given the pivotal role of microbes in ecosystem functioning and the constantly increasing anthropogenic impact on ecosystem, predicting the responses and dynamics of microbial communities to different disturbance regimes has become crucial (Chapin III et al, 2000; Griffiths & Philippot, 2012). Studies on secondary succession can play an instrumental role in understanding the resistance and/or resilience of microbial communities in response to perturbations.

Petroleum hydrocarbon pollution is one of the major anthropogenically introduced disturbances in terrestrial and aquatic ecosystems. As discussed in the previous sections, some components of petroleum hydrocarbons are highly toxic to microbes and therefore will have a negative impact on microbial diversity. On the other hand, oil pollution also provides an increased nutrient input in form of hydrocarbons which can be degraded by various microbial groups. An event of oil pollution can thus trigger a series of successional changes in the structure and composition of microbial communities (Kaplan & Kitts, 2004; Dubinsky et al, 2013; Zhou et al, 2014; Roling et al, 2004). In rhizoremediation of petroleum hydrocarbons, the plant rhizosphere is an additional factor affecting the microbial community structure and can be expected to influence the successional trajectories of microbial communities. Furthermore, plant growth and even seasonal changes may have a considerable role in modifying the rhizosphere associated microbial communities (Smalla et al, 2001; Shakya et al, 2013; Dunfield & Germida, 2003). Taken together, these issues underpin the need for studies which can provide insight into microbial succession in polluted soils and rhizospheres. Secondary succession of bacterial communities in oil contaminated *Populus* rhizosphere was explored first in a greenhouse set-up (**I**) and then under field conditions (**II**) in this thesis.

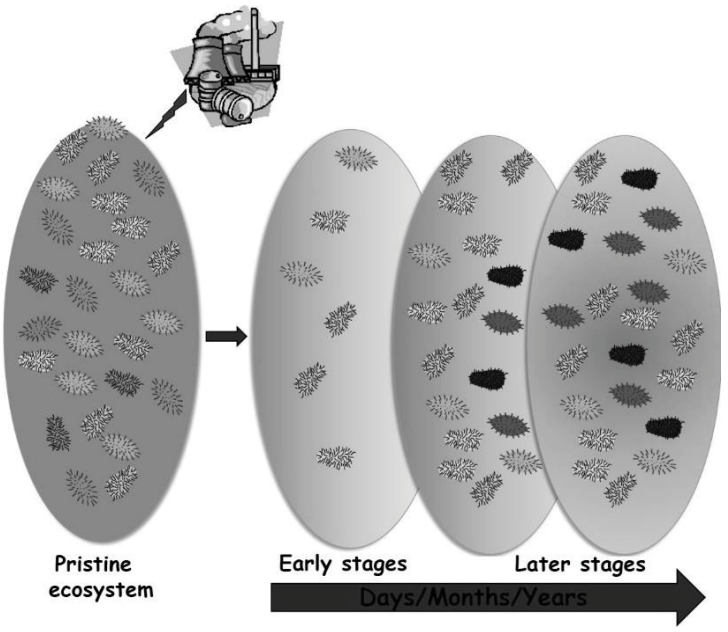


Fig. 5: A schematic representation of successional changes in microbial communities as an aftermath of pollution

1.7. Spatial heterogeneity in polluted sites and geostatistical approaches

Spatial heterogeneity of contaminants and other physicochemical and microbial properties in polluted sites are challenging for various steps of bio-/phytoremediation. Firstly, pre-evaluation or characterization of the extent of pollution and inherent catabolic potential of a site can be complicated as it requires an efficient sampling scheme to cover the spatial heterogeneity in polluted sites (Nedunuri et al, 2000). Secondly, for successful implementation of a suitable remediation regime at a field scale, *i.e.*, up-scaling of bio-/phytoremediation, it is important to consider the spatial patterns displayed by indigenous microbes which may co-vary with the spatial patterns of geo-chemical properties of a polluted site (Törneman et al, 2008; Bengtsson et al, 2010). Last but not least, evaluating the success of a remediation regime according to regulatory guidelines is often based on a point-by-point basis rather than an average of data points from across the site (Jennings & Petersen, 2006). All these issues highlight the necessity of integrating the spatial factor in study designs dealing with bioremediation.

Geostatistics is a powerful tool for gaining insights into such spatial heterogeneity. It includes a set of statistical methods for incorporating the spatial coordinates in data processing, modelling and description of spatial patterns, prediction at un-sampled locations and assessment of uncertainty related to these predictions (Goovaerts, 1998; Goovaerts, 1999). The techniques of geostatistics were initially developed and used in mining, petroleum industry and hydrogeology. Gradually, geostatistics paved its way from the spatial analysis of physicochemical soil properties to studies of spatial patterns of plants (Vieira, 1983; Sutherland

et al, 1991), soil microorganisms (Wollum & Cassel, 1984) and other soil surface organisms (Rossi et al, 1992). In recent years, geostatistics has aided the versatile toolbox of microbial ecologists in deciphering various ecological phenomena associated with spatial scales (Enwall et al, 2010; Wessén et al, 2011; Bru et al, 2010; Philippot et al, 2009; Krause et al, 2009). While geostatistical approaches are often used by environmental agencies and consultancies for the characterization of contaminants of sites, these methods are rarely employed for assessment of microbial community structure and activity on such sites. Geochemical properties that effect the distribution of subsurface microbes do not vary randomly but exhibit spatial continuity (Brockman & Murray, 1997). Some key concepts of geostatistics which are routinely used in description, modelling, prediction and mapping of spatial soil properties and have been applied in article **III** in this thesis are described briefly as follows (Source: (Ettema & Wardle, 2002; Goovaerts, 1998)).

Autocorrelation (spatial dependence): Autocorrelation is the statistical term for heterogeneity in spatial data. It quantifies the resemblance between neighbours as a function of spatial separation distance. When near neighbours are more similar than are far neighbours, data are autocorrelated, violating the assumption of data independence in standard parametric statistics.

Semivariogram and semivariogram modelling: Semivariograms are used in the first steps of spatial prediction. Semivariogram is a function that relates semivariance (or dissimilarity) of data points to the distance that separates them. Its graphic representation can be used to provide a picture of the spatial correlation of data points with their neighbours. Main components of semivariograms include range, sill and nugget (Fig. 6). The distance where the model first flattens out is known as the **range**. Range represents the extent or patch size of heterogeneity. Sample locations separated by distances closer than the range are spatially autocorrelated, whereas, locations farther apart than the range are not. The value that the semivariogram model attains at the range (the value on the y-axis) is called the **sill. Nugget** is indicated by the intercept closer to origin in y-axis; it represents the variance due to sampling error and/or spatial dependence at scales not explicitly sampled. Difference between sill and nugget i.e. **partial sill** indicates the spatially dependent predictability of the property being studied. Higher value of nugget relative to partial sill corresponds to higher "noise" or sampling errors.

Semivariogram modelling involves fitting a semivariogram curve to the empirical data collected. The goal with this step is to obtain the best fit and to incorporate the existing knowledge about the phenomenon being studied into the model; the selected model is further used in making predictions. A vast range of models are available, for example, Circular, Spherical, Tetraspherical, Pentaspherical, Exponential, Gaussian, Rational, Quadratic, Hole Effect, K-Bessel, J-Bessel, Stable etc.; each model is designed to fit different types of phenomena more accurately (Goovaerts, 1998).

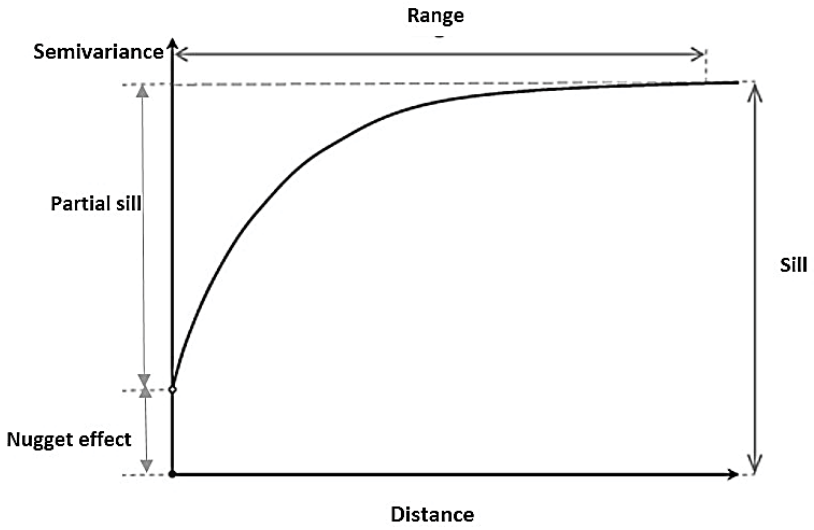


Fig. 6: A theoretical semivariogram

Spatial prediction, Kriging: Estimating soil properties at unsampled locations (spatial prediction) and mapping them are among the most important applications of geostatistics**.** Kriging is an interpolation technique in which the surrounding measured values are weighted to derive a predicted value for an unmeasured location. Weights are based on the distance between the measured points, the prediction locations, and the overall spatial arrangement among the measured points. In this way, kriging serves two different purposes: quantifying the spatial structure of the data and producing a prediction. Kriging was named after the South-African mining engineer Danie G. Krige (Krige, 1976). There are many different kriging algorithms and selection of an applicable algorithm depends on trends in data under study. Examples of these algorithms include- ordinary, simple, universal, probability, indicator, factorial, disjunctive kriging, co-kriging and multivariate factorial kriging (Goovaerts, 1999; Goovaerts, 2001; Oliver & Webster, 1990; Stein, 1999). Selection of a suitable algorithm should be made based on the semivariogram properties and cross-validation before producing a prediction map by kriging. Fig. 7 illustrates some examples of semivariogram models and associated predictive maps.

Spatial scaling of biodiversity remains a central goal in ecology. For a long period, spatial variability in patterns exhibit by soil biota was considered as "noise" or complication in study design and interpretation. It is becoming evident, however, that spatial variability is a key rather than the obstacle in understanding the structure and function of soil biodiversity (Green et al, 2004). By combining molecular methods which provide a high resolution portrait of microbial populations and geostatistical approaches, it might be possible to unravel numerous obscurities regarding the generation and maintenance of soil microbial diversity.

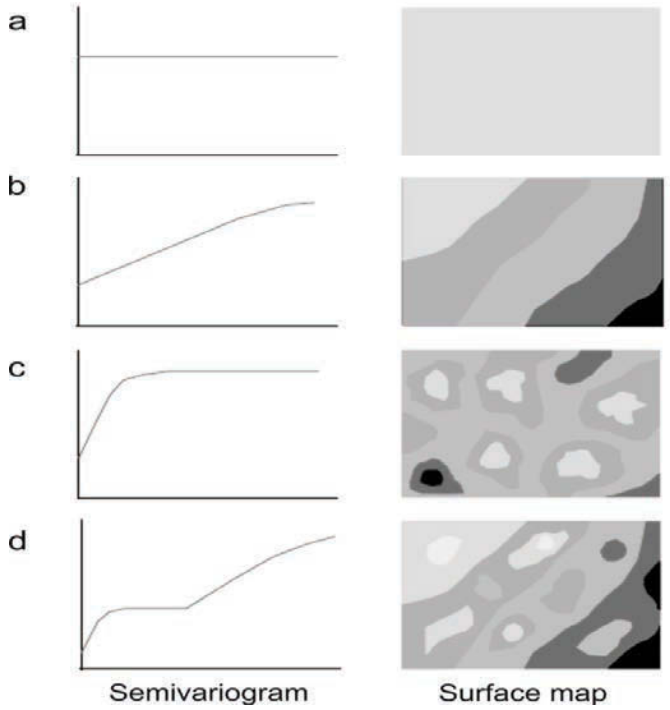


Fig. 7: Hypothetical semivariograms and associated prediction maps illustrating different patterns encountered during spatial analyses. A) Pure nugget effect, Variance is not spatially structured. B) Gradients and large scale heterogeneity. C) Small scale heterogeneity, patchy distribution of hotspots and cold spots. D) Nested heterogeneity of spatial patterns. Source- Ettema & Wardle, 2002

2. Outline and Aims

The main aim of this thesis project was to analyse and interpret temporal and spatial changes in microbial communities in hydrocarbon polluted soils and *Populus* rhizosphere in order to understand the ecological processes regulating these patterns and influencing rhizoremediation. This project was carried out on 3 different levels: a) a short term greenhouse study, b) a 2-year field study and c) a case study on an aged contaminated site (Fig. 8). The specific objectives were:

a) To determine the immediate and long term responses of soil and rhizosphere bacterial communities to hydrocarbon pollution **(I, II & III)**.
b) To explore the co-occurrence patterns of bacterial groups and to understand the possible ecological roles of co-occurring groups during the course of succession **(I &II).**
c) To access the catabolic diversity of bacteria in hydrocarbon contaminated ecosystem in order to understand the structure-function relationship of bacterial communities **(I &II).**
d) To evaluate the abiotic and biotic factors regulating the spatial distribution of microbial communities in an aged polluted site **(III).**

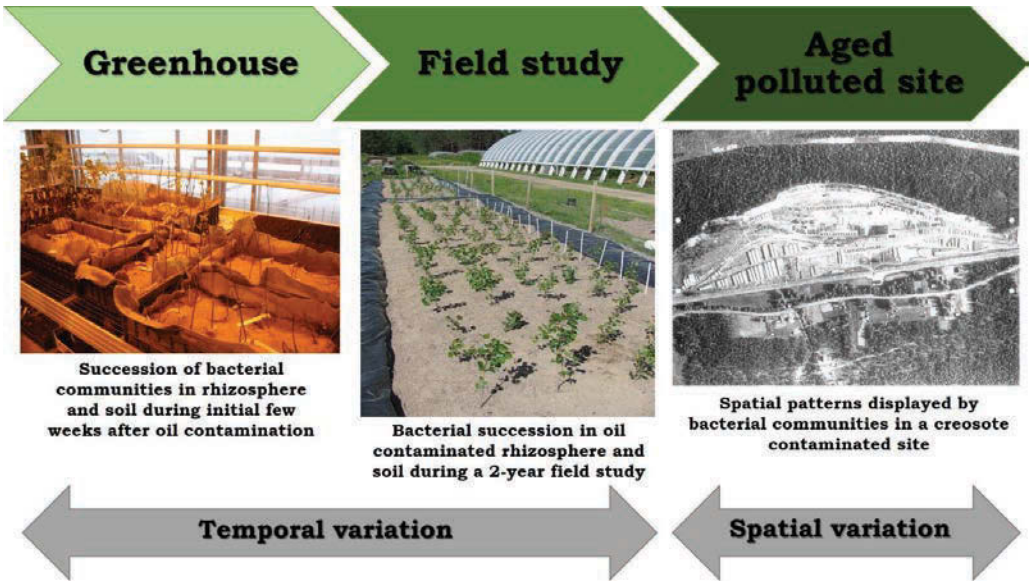


Fig. 8: Outline of the thesis project

3. Materials and methods

3.1.1. Study design and sample collection

A) Short term greenhouse experiment (I)

A 10-week greenhouse experiment was conducted in order to study the successional dynamics of bacterial communities in the *Populus* rhizosphere immediately after oil pollution. This study was conducted at the Finnish Forest Research Institute, Haapastensyrjä Tree Breeding Station in Southern Finland (60°37’24°25′, 1250 d.d.). Hybrid aspen (*Populus tremula* × *Populus tremuloides*) seedlings (20 cm minimum height) were grown in containers (40 × 60 × 12 cm, Takopot TA913, capacity 580 cm3, SCA Packaging, Finland) containing forest mineral soil. Top 25–30 cm of soil from which upper 3–5 cm was removed was used in this experiment. The soil was mixed with heavy fuel oil (Neste Oil ltd.) to an effective 1% concentration and incubated for a week at 18 °C before planting aspen. Microcosms were illuminated using high-pressure sodium lamps (400 W) and the incubation temperature in greenhouse was kept at 18 °C.

Composite soil samples (six sub samples), ~20 g, were taken from rhizosphere soil and bulk soil every week. The rhizosphere soil was obtained by lifting the seedling from the pot, shaking it gently and taking the sample from the remaining soil adhered to the roots. This experiment also included clean controls (same soil without oil addition). Altogether, this experiment included 6 different treatments: oil contaminated rhizosphere, oil contaminated bulk, and oil contaminated unplanted soil, clean rhizosphere, clean bulk and clean unplanted soil.

Fig. 9: Greenhouse study with hybrid aspen planted in oil contaminated (1%) soil.

B) Long term field study (II)

A field study was performed with hybrid aspen (*Populus tremula* × *Populus tremuloides*) planted in oil polluted soil; the sampling time points were planned to take into account the seasonal variation in the boreal climate zone (Fig 10). Soil used in this field study was excavated from an accidental oil spill site located by a motorway in Southern Finland. Following the excavation, the soil was transported to the Haapastensyrjä plant breeding station. After thorough mixing, the soil was planted with hybrid aspen seedlings (minimum 20 cm). In the same plot (3 m × 12 m), a small area (0.8 m× 2.5 m) was left unplanted and was partitioned from the rest of the plot to create an unplanted or bulk soil control. Samples from vegetated and vegetated treatments were taken at 7 time points during 2010-2012. Samples representing rhizosphere associated soil were collected by taking the 6 subsamples around one plant (10 cm below the ground and along 5-7 cm radius of the main root system); 2-3 replicate plants were sampled at each time point. The un-vegetated soil was sampled from the control box (10 cm below the ground and 2-3 replicates per time point).

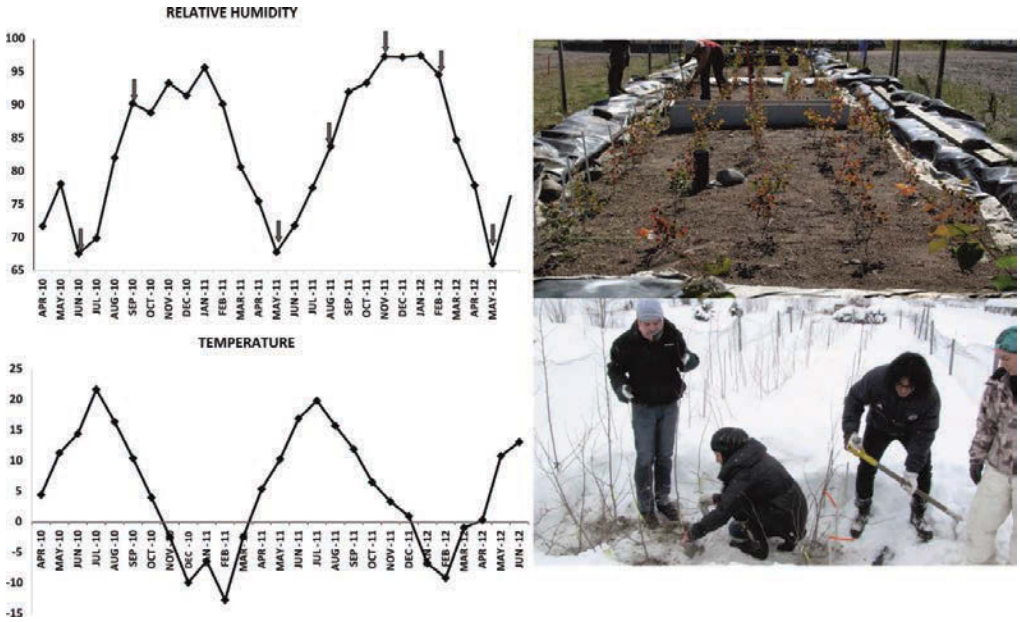


Fig. 10: 2-year field study with hybrid aspen planted in oil contaminated soil. Left panel represents the relative humidity and temperature variation during the study period and grey arrows in the relative humidity graph indicate the sampling time points.

C) Aged creosote-contaminated site (III)

An aged creosote-contaminated site located in South-eastern Finland (60° 55' 00" N and 27° 26' 00" E, Luumäki) was studied. This site was a wood preservation facility located in South-eastern Finland where creosote had been used to impregnate railroad sleepers between 1947 and 1958, resulting in high concentrations of creosote in the soil. As per the previous site investigations by Golder associates about ~ 1.3 hectares area in the central part, where the treatment facility was located, was characterized to be heavily contaminated. A sampling grid was created for the site (Fig. 11). The central area which was anticipated to be more contaminated was sampled using a 20 m x 20 m grid. Surrounding, less contaminated areas were investigated using a 40 m x 40 or 20 m x 80 m grid. In each plot, five 0.5 m deep sampling pits were excavated with a shovel. Top soil was collected from 0-0.1 m, and samples from five pits (four from the corners of the plot/square and one from centre) were combined to form composite samples representing each plot. Approximately 0.25 l of soil was collected from each pit and combined as 1 l composite sample in a gas tight plastic sampling bag. The composite sample was mixed well in the bag and then divided into portions for different analyses.

Fig. 11: Sampling design for studying the spatial patterns of microbial diversity and activity at Luumäki site. The black rectangular box in the middle represents the plots which were chosen for the microbial analysis. In addition, few plots outside the heavily contaminated area were also included in the study (marked by filled black squares). The hollow circles in each plot represent the sampling points.

3.2. Methods

The methods used in analysis of structural and functional microbial communities, microbial activity and soil chemical parameters have been described in the original articles (**I, II & III**) and are listed in the table below.

Method	Described and used in:
Molecular methods	
DNA extraction from rhizosphere and soil	**I, II & III**
PCR amplification of 16S rRNA, *edo* (I.3.E) and *alk*B genes	**I, II & III**
T-RFLP fingerprinting of 16S rRNA gene communities	**I**
T-RFLP fingerprinting of *edo* gene communities	**I**
Cloning	**I**
Sanger sequencing of 16S rRNA clones	**I**
454 pyrosequencing of 16S rRNA gene amplicons	**II & III**
454 pyrosequencing of *edo* gene amplicons	**II**
454 pyrosequencing of *alk*B gene amplicons	**II**
Microbial activity assays	
Most probable number (MPN) count for hydrocarbon degraders	**I**
Basal respiration assay	**III**
Fluorescein diacetate (FDA) hydrolysis assay	**III**
Soil chemical analyses	
Macro- and micro nutrients	**II & III**
Total petroleum hydrocarbons (TPH)	**II & III**
16 USEPA priority PAHs	**III**
Heavy metals	**II & III**
pH	**I, II & III**
Sequence analyses (methods and software)	
T-RFLP electropherograms - GeneScan (Applied Biosystems)	**I**
Quality check of clone library sequences – Genedoc	**I**
In silico restriction digestion – Sequence manipulation suit	**I**
Sequence alignment – Clustal W	**I, II**
Basic local alignment search tool (BLAST)	**I, II, III**
Phylogenetic analysis - MEGA	**I, II**
Phylogenetic trees - iTOL	**II**
16S rRNA pyrosequencing data analysis - MOTHUR	**II & III**
edo and *alkB* pyrosequencing data analysis- MOTHUR	**II**
Distance matrix of translated functional genes sequences -PHYLIP	**II**
Statistical analyses (methods and software)	
Multivariate analysis (PCA, RDA, NM-MDS, NPMANOVA, Biplot analysis) – PAST & R statistical package	**I, II & III**
Hierarchical clustering – Cluster 3 & Java tree view	**I & II**
Diversity indices - PAST	**I, II & III**
Correlation – Graphpad Prism	**III**
Geostatistical modelling - ArcGIS	**III**
Spatial prediction (Kriging) - ArcGIS	**III**

4. Results and Discussion

This chapter provides an overview and discussion on key findings of the thesis. The detailed description with figures and tables, referred to in the following text, is presented in the original articles (**I, II & III**) of this thesis.

4.1. Secondary succession of bacterial communities in oil pollution (I & II)

Oil pollution causes a disturbance in the chemical and physical soil environment thereby altering the ecological niches available to soil biota. Soil microbes have the capacity to utilize hydrocarbons as a substrate. Nevertheless, depending on the level of pollution (concentration), oil compounds are also toxic to microbes. Followed by oil pollution, a series of changes (secondary succession) can be triggered in the microbial community structure. We studied the successional trajectories of bacterial communities in oil polluted soil planted with aspen in two different studies: i) a short-term greenhouse study and ii) a long-term field study.

4.1.1. Temporal patterns of bacterial communities during a 10-week greenhouse experiment (I)

We performed 16S rRNA T-RFLP fingerprinting of bacterial communities from contaminated and uncontaminated rhizosphere, bulk and unplanted soil samples collected weekly. A sudden decrease in soil bacterial diversity (2.2 to 1.4; Shannon indices) was noticed one week after the oil amendment. In the following weeks, an increase in diversity was observed in the oil rhizosphere, bulk and oil control (unplanted) but oil rhizosphere maintained a higher diversity compared to the other treatments (supplementary table 1**, I**). Most probable number (MPN) assay revealed an increase in the abundance of culturable oil-degraders in the polluted rhizosphere during the first 2 weeks (Fig 6, **I**). Initial decrease in diversity reflected the immediate disturbance caused by oil contamination and its deleterious effect on bacterial communities, which is typical in secondary succession (Horn, 1974). In the later weeks, however, the bacterial communities started to adapt to the oil contamination as noticed from the increasing diversity. Higher diversity of bacterial communities along with the increased number of oil degraders in rhizosphere samples indicated a rhizosphere effect introduced by aspen plants in oil contaminated soil.

Multivariate analysis (PCA) of bacterial community structure (Fig. 12) further indicated a clear effect of oil pollution on the bacterial communities as samples from oil contaminated treatments were separated from uncontaminated ones along PC1 (45.6% variance). A non-random or directional pattern of temporal development was observed in oil-polluted rhizosphere, bulk and unplanted bacterial communities during the course of study. Also, the temporal patterns observed in the contaminated treatments were alike, with oil bulk and oil control showing similar community structure during the time course. In contrast, bacterial communities in uncontaminated treatments showed random temporal dynamics with much less variation with time compared to the contaminated treatments. These results clearly indicated the successional changes initiated by oil pollution. While slight differences between rhizosphere, bulk and unplanted controls could be detected among the contaminated treatments, effect of oil pollution on bacterial community structure was more pronounced than the

rhizosphere effect. A recent study also reported similar findings; they noticed a lower impact of willow rhizosphere compared to hydrocarbon contamination levels on microbial community structure (Yergeau et al, 2014).

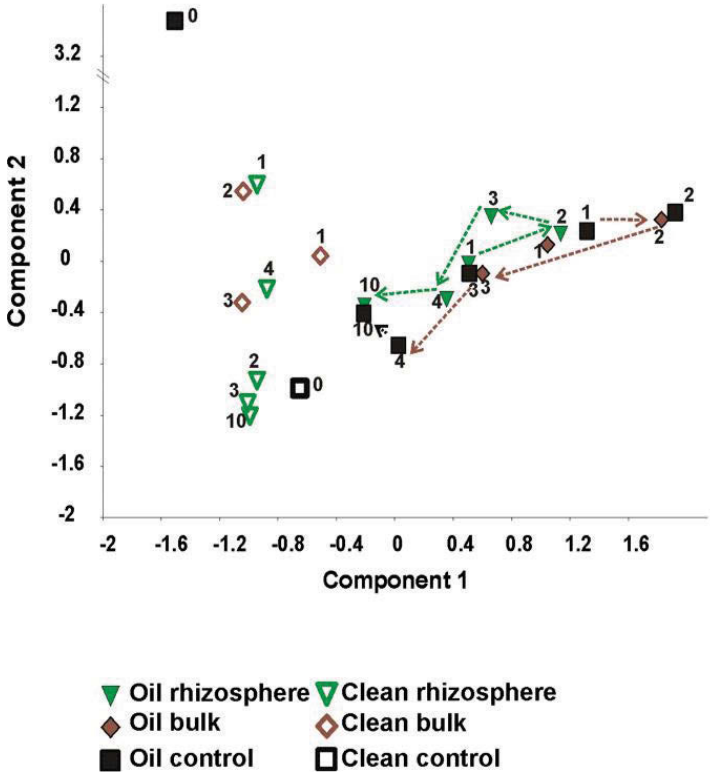


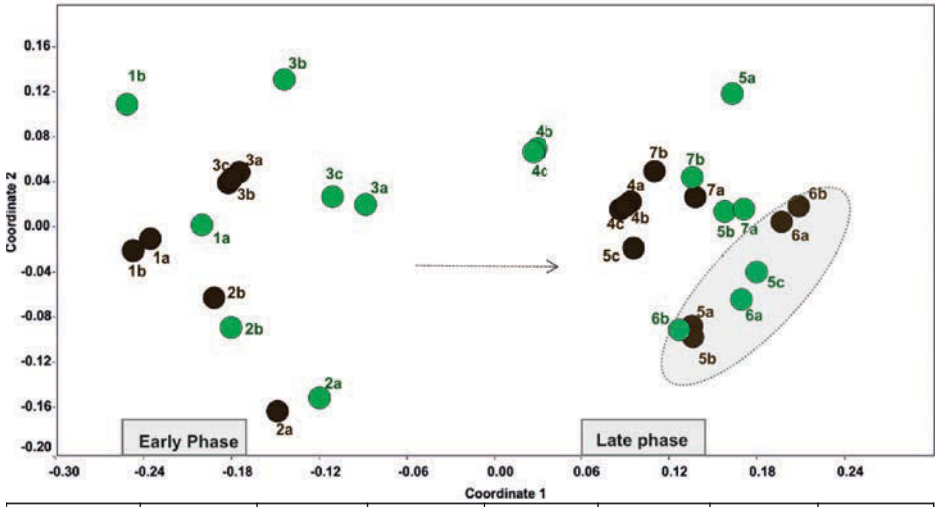
Fig. 12: Principal component analysis based on 16S rRNA gene T-RFLP fingerprinting of bacterial communities. Numbers in the sample code represent the week number when sampling was performed. Colour codes below the figure represent different treatments in this study.

4.1.2. Temporal patterns of bacterial communities during a 2-year field study (II)

We further aimed to analyse the long-term successional patterns of bacterial communities in oil-polluted aspen rhizosphere and soil in a field experiment. To this end, we sampled rhizosphere-associated and un-vegetated soil at 7 different time-points during 2 years; the sampling time-points were chosen to take into account the seasonal variations. 454 pyrosequencing of 16S rRNA gene amplicons was performed to assess the bacterial community dynamics. A total of 3215 OTUs (97% sequence similarity) were detected from 34 samples after removing the singletons. Two distinct clusters of bacterial communities (NPMANOVA; p=0.0001) were separated in multivariate analysis of 16S rRNA OTUs, referred to as early and late phase in the following text and figures (Fig. 13). Bacterial communities in contaminated rhizosphere and bulk (un-vegetated) soil from June 2010, September 2010 and May 2011

grouped in the early phase, whereas those from August 2011 to February 2012 grouped in the late phase cluster. While differences in rhizosphere and bulk communities from the same time points could be observed, sampling time points had a more prominent effect on bacterial community structure. Biological replicates from rhizosphere displayed a higher variation compared to biological replicates of bulk soil. During the late phase, bacterial communities in winter months (indicated as a grey ellipse in Fig. 13) showed marked variation (NPMANOVA p=0.0005) from spring and summer communities.

It has earlier been reported that during the course of oil degradation, due to differential use of hydrocarbon components, distinct successional changes occur in soil bacterial communities (Kaplan & Kitts, 2004; Röling et al, 2002; Yu et al, 2011; Roling et al, 2004) . We were able to notice clear successional changes in bacterial communities in oil polluted *Populus* rhizosphere and soil during the 2-year field study. We could not notice significant differences between rhizosphere and bulk diversity indices. A rise in diversity in both rhizosphere and bulk could be noticed at the second sampling time point. These results are similar to our short-term greenhouse study where the bacterial diversity increased noticeably after the initial drop indicating resilience of bacterial communities (**I**). Another similar observation in both these studies of secondary succession was a relatively higher effect of oil pollution compared to the rhizosphere effect on the bacterial community structure. Additionally, in this 2-year study, seasonal variation was shown to play a role in regulating the bacterial community structure during succession.



Sample code	1a, 1b	2a, 2b	3a, 3b, 3c	4a, 4b, 4c	5a, 5b, 5c	6a, 6b	7a, 7b
time-point	June 2010	Sep 2010	May 2011	Aug 2011	Nov 2011	Feb 2012	May 2012

Fig. 13: Non-metric multidimensional scaling (NM-MDS) based on Bray-Curtis distances of 16S rRNA OTUs (97% sequence similarity) from oil polluted rhizosphere and soil. Sample codes are mentioned below the NM-MDS plot. In the sample codes, a, b &c represent biological replicates. Rhizosphere samples are represented by green filled circles and un-vegetated soil samples are represented by brown filled circles.

4.1.3. Co-occurrence patterns of bacterial groups during succession (I&II)

We explored co-occurrence patterns of OTUs during the successional processes in both of our rhizoremediation studies (greenhouse and field experiment). Non-random co-occurrence patterns have been useful in unravelling the ecological processes governing community assembly rules in macro- and micro-ecology (Ulrich & J Gotelli, 2007; Gotelli & McCabe, 2002; Ruan et al, 2006; Barberán et al, 2012; Horner-Devine et al, 2007). It has also been suggested that analysing co-occurrence patterns varying with environmental parameters could give an insight into community structure-function relationships (Fuhrman, 2009). Monitoring the behaviour of co-occurring groups in relation to specific environmental factors such as pollution, influence of rhizosphere and time helped us to infer their possible ecological roles. Hierarchical clustering is a traditional approach in ecological studies to reveal relationships between communities sampled from different environments. We performed the hierarchical clustering of OTUs (instead of communities) based on their co-occurrence patterns across the whole time-series and in different treatments.

In our short term greenhouse study, we noticed two different linkage groups or clusters of 16S rRNA gene OTUs (T-RFs) (Fig. 4, **I**). Cluster A was composed of a group of bacteria present in all treatments: clean and oil-contaminated rhizosphere, bulk and unplanted soil, at all time points throughout the study but relatively more dominant in the clean treatments. Based on the *in silico* digestion of clone library sequences, some of the T-RFs were identified as Alphaproteobacteria (T-RF 65 and 152), Acidobacteria Gp I (T-RF 95 and 268) and Bacteroidetes (T-RF 92). These groups probably represented the generalists or k-strategists in oil pollution. While Alphaproteobacteria and Bacteroidetes contain some well-known degraders of oil components, Acidobacteria possibly represented the oil-tolerant groups in this study. Cluster B consisted of bacterial groups which were highly dominant in the oil-contaminated treatments and could hardly be observed in the clean treatments; most of the T-RFs belonging to this cluster were identified as Betaproteobacteria. This cluster represented specialists or oil degraders in oil-polluted soil and rhizosphere.

Next, we analysed co-occurrence patterns of bacterial groups in our long-term field study. 454 pyrosequencing of 16S rRNA gene amplicons used in this study provided a higher resolution and an improved identification of OTUs. Hierarchical clustering of 16S rRNA gene OTUs brought forward two major co-occurring groups: cluster A dominating in the early phase of succession and cluster B dominating in the late phase of succession (Fig 4, **II**); see section 4.1.2 for the clarification of early and late phases. The identified OTUs in early phase cluster belonged to Actinomycetales, Sphingomonadales, Rhizobiales, Caulobacterales, Burkholderiales and Xanthomonadales. The late phase communities were dominated by OTUs affiliated to unclassified Gammaproteobacteria, unclassified Betaproteobacteria, Rhizobiales, Acidobacteria GpI, Acidobacteria Gp4 and unclassified bacteria. Subcluster 3, which was a part of the late phase cluster represent a group of bacteria which were also observed in the early phase rhizosphere communities. Members of subcluster 3 belonged to Sphingomonadales (*Parvibaculum*), unclassified Beta and Gammaproteobacteria and Acidobacteria Gp4. Apart from these early and late phase members, two OTUs affiliated to the order Rhizobiales could be detected all along the study duration. This analysis enabled us to identify the bacterial groups dominating in the early and late phase of secondary succession. By comparing the co-occurrence patterns of 16S rRNA gene OTUs with those displayed by catabolic gene OTUs (alkane hydroxylases and extradiol dioxygenase), we were further able to infer the possible

catabolic functions of community members at different successional stages. Community dynamics and co-occurrence patterns of catabolic communities are discussed in the following sections.

4.1.4. Catabolic genes in oil pollution (I&II)

Two different marker genes were used for studying the bacterial catabolic diversity in oil-polluted rhizospheres and soils: extradiol dioxygenase as a marker for degraders of aromatics and alkane monooxygenase as a marker for degraders of aliphatics.

A. Extradiol dioxygenases: community dynamics and diversity (I&II)

Extradiol dioxygenases catalyse the *meta*-cleavage of aromatic ring as the third step in the peripheral degradation pathway of aromatic compounds. These enzymes have been characterized in Alpha-, Beta-, Gammaproteobacteria and Actinomycetales and have been shown to degrade a broad range of aromatics including biphenyls, naphthalene, phenanthrene, anthracene, fluoranthene, chrysene, naphthalenesulfonates, 6-dimethyldibenzothiophene tetralin, dibenzothiophene and ethylbenzene (Pinyakong et al, 2003; Andujar et al, 2000; Story et al, 2001; Lu et al, 2000; Keck et al, 2006; Jeon et al, 2006; McLeod et al, 2006).

T-RFLP fingerprinting of *edo* gene communities was performed to monitor the population of aromatic degraders in our greenhouse study (**I**). Marked difference in *edo* gene communities of contaminated and clean treatments was observed (Fig 1b, **I**). Distinct temporal variations were detected in the oil contaminated treatments and also in the clean rhizosphere. Temporal variations of extradiol dioxygenase communities in the clean rhizosphere could be due to the secretion of secondary metabolites which resemble some aromatics, thereby stimulating the aromatic degraders. Hierarchical clustering of T-RFs revealed 3 important co-occurring clusters. These T-RFs clustered together based on their similar temporal dynamics and occurrence patterns in different treatments (Fig. 5, **I**). Cluster X (T-RF 62, 64, 67, and 69 bp) represented the T-RFs dominant in the clean treatments and rarely present in the contaminated treatments. Cluster Y (56, 53, 77, 73, and 80 bp) represented T-RFs present in both the clean and contaminated treatments. Cluster Z (330, 319, 384, 465, 395, 461, and 463 bp) consisted of the T-RFs clearly dominant in the polluted treatments. Co-occurrence patterns of extradiol dioxygenase OTUs were comparable to the 16S rRNA genes in this short-term greenhouse study.

We performed 454 pyrosequencing of extradiol dioxygenase amplicons from 34 samples taken at 7 time points from rhizosphere and un-vegetated soil during the 2-year study period (**II**). A total of 22391 good quality sequences were clustered into 153 EDO OTUs (90% amino acid sequence similarity). Similar to the 16S rRNA communities, a clear succession was displayed by EDO communities (Fig. 3a, Article II). Differences in rhizosphere and bulk communities were more prominent in the late phase of succession. We also observed a steady rise in EDO diversity (Shannon indices, supplementary figure 3, **II**) during the course of succession.

Co-occurrence patterns of EDO OTUs were determined by hierarchical clustering to differentiate between the members of late phase and early phase communities (Fig. 5, **II**). In order to identify the putative hosts of the extradiol dioxygenase sequences detected in this study, we constructed a phylogenetic tree with the OTU representative sequences; closest hits

from NCBI database (-nr) and sequences from different clusters of the EDO tree from a study by Sipilä *et al.* 2008 were included as reference sequences. Members of early phase communities grouped in 2 different clusters of EDO phylogenetic tree (Fig 6, **II**). The cluster harbouring some of the most abundant OTUs of early phase had representative sequences from various genera of Sphingomonadales; these group of enzymes, characterized in *Sphingomonas* species, have been reported to target a diverse set of polyaromatic compounds (Jouanneau & Meyer, 2006). Other members of early phase community belonged to a phylogenetic cluster with no cultured representatives.

In the late phase of succession, community composition of EDOs was much more diverse and the sequence dissimilarity between the members was higher according to the EDO tree. OTUs dominating in late phase showed sequence similarity to *Sphingomonas, Sphingopyxis, Novosphingobium, Burkholderia, Cycloclasticus, Polaromnas, Ralstonia, Pseudomonas and Rhodococcus* type extradiol dioxygenases. In addition to these cultured representative from which extradiol dioxygenase enzymes have already been characterized, we also found high sequence similarity of OTUs in our study with sequences from Alphaproteobacterial species *Parvibaculum* and *Sandarakinorhabdus.* However, neither the EDO enzymes have been purified and characterized from these strains, nor their degradative capacities towards PAHs have been tested. *Parvibaculum lavamentivorans* has been shown to play a role in the degradation of surfactants such as alkylbenzenesulfonates (Schleheck et al, 2004; Dong et al, 2004; Schleheck et al, 2011). Sequence from *Sandarakinorhabdus sp.* Strain AAP62 was in the same phylogenetic cluster as *Parvibaculum* and the genome of this photoheterotrophic fresh water strain has recently been sequenced (Zeng et al, 2013). High sequence similarity with the extradiol dioxygenase sequences from our study indicates the presence of these genes in these two strains; characterization of degradative capacities of these strains and efforts for isolation of respective enzymes would therefore be useful. Many clusters with no representatives from cultured strains were observed in our EDO phylogenetic tree (Fig. 14); these clusters indicate the tremendous unexplored diversity of these enzymes in contaminated soil and rhizosphere.

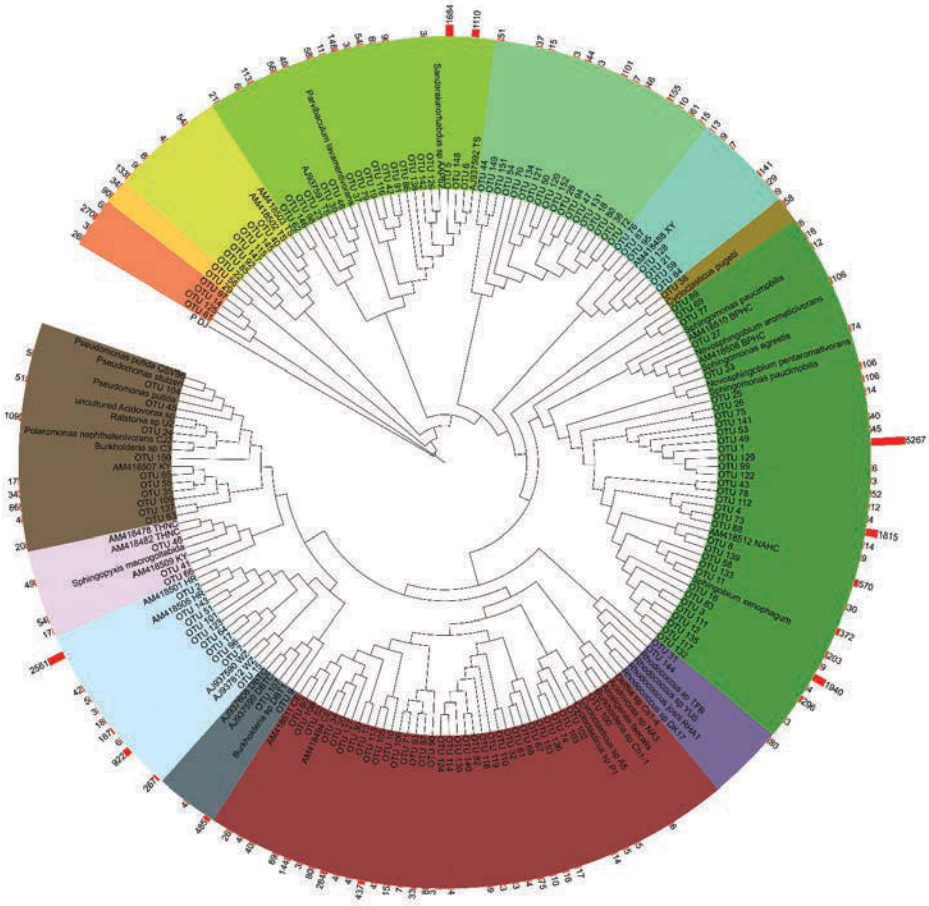


Fig. 14: Maximum likelihood tree of extradiol dioxygenase (EDO) amino-acid sequences based on the JTT model (Jones et al, 1992) . The bootstrap consensus tree was inferred from 500 replicates. One representative sequence from each EDO OTUs was included in the tree along with the closest hits from NCBI non-redundant protein database. OTUs with more than 2 sequences were chosen for better visualization. The red bars indicate the abundance of OTUs (number of sequences).

B. Alkane monooxygenases: community dynamics and diversity (II)

454 pyrosequencing of alkane monooxygenase amplicons was performed in order to reveal the community composition and dynamics of alkane degrading bacterial populations in the long-term field study. Primers used in this study are known to have a broad coverage of bacterial phyla making them suitable for community ecology studies (Jurelevicius et al, 2013; Kloos et al, 2006). A total of 53418 good quality sequences were obtained from 34 samples. After removing the singletons, 603 AlkB OTUs (95% amino-acid sequence similarity) were detected. Similar to the 16S rRNA communities, multivariate analysis (PCA) revealed the separation of alkane monooxygenase communities into early and late phases thereby showing a clear successional change. In the early phase, AlkB communities in rhizosphere showed marked differences from the bulk/unplanted communities (NPMANOVA p=0.0003). Hierarchical clustering of top 50 AlkB OTUs revealed three major co-occurrence patterns based on their

temporal behaviour (Fig 7, **III**). Cluster 1 was composed of OTUs which dominated in the early phase bulk and rhizosphere and thereafter underwent a more prominent decline in the late phase rhizosphere. Cluster 2 consisted of OTUs dominating in the late phase and Cluster 3 was composed of OTUs which could be observed throughout the study with some variation in relative abundances. These patterns were comparable to those displayed by the structural communities (16S rRNA). We noticed a considerable rise in diversity and decrease in dominance in AlkB communities during the course of succession (supplementary Fig 3, **II**).

AlkB communities in oil polluted *Populus* rhizosphere and soil were highly diverse and belonged to a broad range of taxonomic groups including Alpha-, Beta-, Gammaproteobacteria, Actinobacteria and Bacteroidetes (Fig. 15). The databases of alkane monooxygenases are far more extensive than extradiol dioxygenases thereby providing a better identification of hosts. Interestingly, the phylogeny of AlkB sequences did not correspond to the taxonomical closeness of the bacterial groups harbouring them. With an exception of few clusters in the AlkB tree, which were dominated by either Proteobacteria or Actinobacteria, sequences from different classes and phyla grouped together in the same clusters. These results were corroborated by a recent study by Giebler et al (2013b) ; they found that *alkB* and 16S rRNA gene derived phylogenies are not always congruent. This is possibly due the extensive horizontal gene transfer (HGT) of alkane monooxygenases among taxonomically distant groups which has been pointed out earlier (van Beilen et al, 2001; Heiss-Blanquet et al, 2005; Van Beilen et al, 2004). Furthermore, multiple copies of these genes might occur within a single organism (van Beilen & Funhoff, 2007). It has been proposed that the clustering of AlkB sequences in a phylogenetic tree might be based on their substrate specificity rather than the phylogenetic origin of bacterial groups carrying these genes (Heiss-Blanquet et al, 2005; Van Beilen et al, 2003).

It is noteworthy that most of the members of early phase AlkB communities belonged to the same cluster in the phylogenetic tree (Fig 8, **II**) indicating phylogenetic closeness and similar substrate specificity of these groups. These early phase members showed a high sequence similarity to *Rhodococcus sp*. MS103a (83 – 91% amino acid sequence similarity) and also to *Pedobacter sp.* MS245e (85%). These observations are congruent with the dominance of Actinomycetales in the early phase 16S rRNA gene communities and suggest the potential role of Actinomycetales as alkane degraders in the early stages of oil pollution. In the late phase however, AlkB communities were more diverse as they spanned across 9 different clusters in the phylogenetic tree. These findings probably point towards the increased diversity related to substrate specificity for alkanes in the later stages of succession.

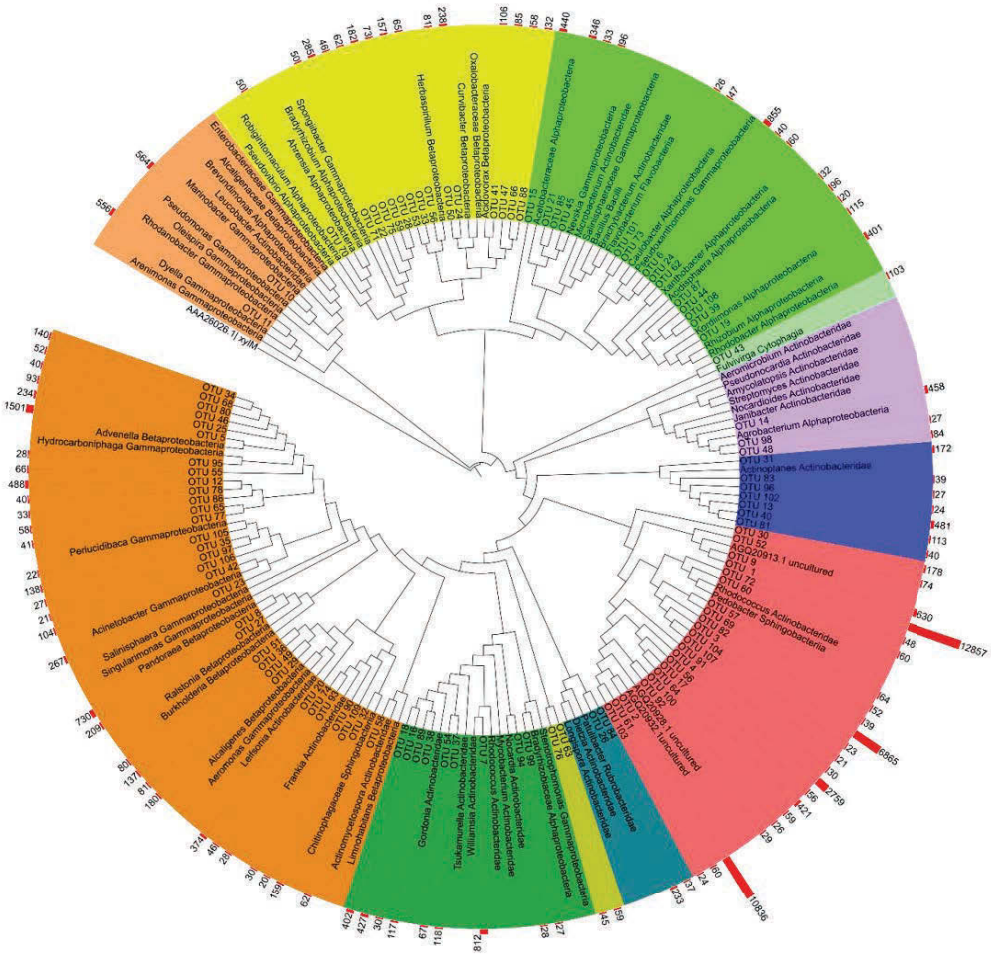


Fig. 15: Maximum likelihood tree of alkane monooxygenase (AlkB) amino-acid sequences based on the JTT model (Jones et al, 1992). The bootstrap consensus tree was inferred from 500 replicates. One representative sequence from each AlkB OTUs was included in the tree along with the closest hits from NCBI non-redundant protein database. OTUs with more than 20 sequences were chosen for better visualization. The red bars indicate the abundance of OTUs (number of sequences).

4.2. Spatial patterns in the creosote-contaminated site (III)

We studied an aged creosote-contaminated site in order to elucidate the spatial patterns of microbial diversity and activity and to relate them with the soil-chemical parameters on the site.

4.2.1. Niche differentiation explaining the spatial patterns of bacterial diversity

We used an intensive grid based sampling scheme followed by 454 pyrosequencing of 16S rRNA gene amplicons and geostatistical modelling in order to relate the spatial patterns of soil

chemical parameters and bacterial diversity at the studied creosote-polluted site. Kriged maps revealed two distinct patterns in the soil chemistry of the site (Fig. 1**, III**): a) patchy distribution of pollutants (TPHs and PAHs) and b) east to west pH gradient (7.8 - 5). Also, bacterial community structure in the polluted site was mainly regulated by the level of pollution and pH (Fig. 2, **III**). Bacterial diversity was found to be significantly lower in the hotspots of pollution (Spearman's r= -0.45, p= 0.008).

We further tried to analyse whether the spatial distribution of bacterial taxa could be related to the spatial variation of soil properties. Semivariogram modelling and mapping of relative abundance of bacterial taxa provided insights into the patterns of niche partitioning at different taxonomic levels regulated by the concentration of creosotes and pH levels. Proteobacteria displayed a patchy distribution pattern and dominated in the hotspots of pollution. Patchiness in spatial distribution was also displayed by Actinobacteria, TM7, Armatimonadetes and Planctomycetes but the relative abundances of these groups were significantly reduced in the hotspots of pollution (Fig 3, **III**). On the other hand, spatial patterns of Acidobacteria, Verrucomicrobia and Bacteriodetes were found to be related to the pH gradient. A considerable dominance of Proteobacteria over other phyla in the hotspots of pollution is supported by the fact that this phylum consists of large number of well-known hydrocarbon degraders. However, the drastic decrease in actinobacterial abundance in the hotspots of pollution contradicts our knowledge of the robust catabolic capacities of actinobacterial groups (Kanaly & Harayama, 2000; Larkin et al, 2005; Arenskötter et al, 2004) and some previous findings that have reported the dominance of Actinobacteria in hydrocarbon polluted soil (Antizar-Ladislao et al, 2008; Johnsen et al, 2007). Interestingly, a similar study on an aged creosote polluted site in Sweden has also reported a decline in actinobacterial abundance in the hotspots of pollution (Törneman et al, 2008). These findings indicate that Actinobacteria were outcompeted by Proteobacteria over time in this polluted site.

We also looked into bacterial responses to environmental factors at lower taxonomic levels (Fig 4, **III**). Among the proteobacterial classes, Betaproteobacteria and Gammaproteobacteria had increased relative abundances in the hotspots of pollution while Deltaproteobacteria showed an opposite trend. Spatial distribution of Alphaproteobacteria could not be explained by any of the measured soil chemical parameters. The tremendous diversity of niche specialization patterns within alphaproteobacterial orders is well known (Ettema & Andersson, 2009). Alphaproteobacterial orders, Sphingomonadales and Rhodospirillales were more abundant in high pollution level, whereas, Rhizobiales followed the opposite trend. pH also played an important role in regulating the spatial patterns of proteobacterial groups dominating in high pollution. Sphingomonadales prevailed in high pH whereas Rhodospirillales preferred lower pH zones in the polluted site. Similarly, betaproteobacterial family Burkholderiaceae was found in higher abundances in low pH and Pseudomonadaeceae (Gammaproteobacteria) were prevalent in higher pH. Several genera of Rhodospiralles have previously been linked with hydrocarbon degradation at moderately acidic pH levels (Röling et al, 2006; Hamamura et al, 2005). Although, Burkholderiaceae and Pseudomonadaceae are among some of the most well-known hydrocarbon degraders in a variety of soils (Leys et al, 2004; Pérez-Pantoja et al, 2012; Silby et al, 2011; Stolz, 2013), the pH optima of these groups is in polluted soils is still unaccounted for. Our results provide some clues about the pH-specific niche preferences of these groups in aged hydrocarbon contamination. Acidobacterial classes displayed contrasting patterns of pH optima in the studied site. Acidobacteria Gp1, Gp2 and Gp3 were favoured in low pH, whereas Acidobacteria

Gp 4 and Gp 6 prevailed in higher pH. These observations are in line with the findings from some recent studies (Foesel et al, 2013; George et al, 2011; Rousk et al, 2010). Acidobacteria came across as generalists in hydrocarbon polluted soil as they appeared to remain unaffected by the varying pollution levels. It is worth mentioning here that role of Acidobacteria as generalist group in pollution was also highlighted in the other two studies (**I&II**). Combining the results of spatial patterns of bacterial diversity with soil chemistry not only provided a thorough knowledge of long term effects of creosote pollution but also brought forward the possibility of utilizing the spatial patterns displayed by specific bacterial groups as proxies in monitoring polluted sites.

4.2.2. Spatial patterns of microbial activity

Assessment of microbial activity in the polluted site was performed by basal respiration assay and fluorescein diacetate (FDA) hydrolysis assay. Basal respiration assay has been found to be a relevant measure of total biological activity in soils and has previously been used as a parameter for monitoring hydrocarbon bioremediation (Margesin et al, 2003; Tuomi et al, 2004). FDA hydrolysis provides a general measure of soil hydrolytic activity as it measures the activity of a broad range of enzymes (proteases, lipases and esterases) capable of cleaving the fluorogenic FDA substrate into fluorescein (Schnurer & Rosswall, 1982); this assay has also been used in hydrocarbon bioremediation studies (Song & Bartha, 1990; Wang & Bartha, 1990; Margesin et al, 2003). The basal respiration rate in the polluted site varied from 0.1 to 1.1 µl CO_2/g soil/ hour and the FDA hydrolysis rate was in the range of 19.6 to 140.5 µg fluorescein/ g soil 3h (Fig 5, **III**). Both measures of microbial activity showed a high correlation with each other ($r=0.75$, $p<0.0001$) and were positively correlated to the concentration of different PAHs and TPHs (Supplementary Table S3, **III**). Higher microbial activity and reduced diversity in the most polluted zones in this aged contaminated site could be an indication of adaptation or enrichment of specific indigenous microbes such as proteobacterial groups dominating in pollution and successful utilization of hydrocarbons as a food source by these groups.

5. Conclusions

Secondary succession and spatial patterns of microbes in hydrocarbon contaminated ecosystems (soils and *Populus* rhizosphere) were the two main topics explored in this thesis. Poplars have been extensively used for rhizoremediation of organic contaminants but surprisingly few studies have focused on its rhizosphere-associated microbial communities in polluted soils.

It was evident from both studies on secondary succession that oil pollution initiated deterministic patterns of temporal changes in soil and rhizosphere-associated bacterial communities. Structural and functional bacterial communities were resilient to oil pollution as shown by the steady rise in diversity after the initial deleterious effect of hydrocarbon components. In the long-term study of secondary succession, distinct changes occurred in the composition of structural (16S rRNA) and catabolic (extradiol dioxygenases and alkane monooxygenases) communities in the early and late phases. A transition from low diversity-high dominance community to high diversity-low dominance groups occurred in the late phase of succession. *Sphingomonas* type extradiol dioxygenases and *Rhodococcus* type alkane monooxygenases dominated the early phase of succession whereas the late phase members of both the catabolic communities were phylogenetically diverse.

Among the two catabolic genes studied, alkane monooxygenase databases were far more developed compared to those of extradiol dioxygenases. The identification of putative hosts of *alkB* sequences was, however, not so straight forward due to the incongruence of *alkB* and 16S phylogenies, most probably caused due to the extensive horizontal gene transfer. It was hard to draw conclusions about the congruence of 16S rRNA and extradiol dioxygenases based phylogeny as very few clusters found in our study contained cultured representatives. This underpins the need for much extensive isolation of aromatic degraders and characterization of enzymes involved in the upper *meta*-pathway of aromatic degradation.

Niche differentiation based on creosote concentration and pH was an important mechanism explaining the spatial patterns of bacterial taxa in the aged creosote contaminated site. Proteobacterial groups were the key groups dominating in high levels of creosote contamination. To our surprise, Actinobacteria, a phyla which consists of many robust hydrocarbon degraders, dramatically decreased in abundance in high creosote pollution. Interestingly, in our 2-year field study of bacterial succession, abundance of actinobacterial groups was also considerably reduced in the late phase of succession. Taken together, these findings lead us to suggest that actinobacterial groups might be the r-strategists in hydrocarbon polluted soils and are less important in the later stages of secondary succession. According to the results obtained in this thesis, Acidobacteria represent the generalists in hydrocarbon contaminated soils.

Geostatistical approaches hold a great promise for characterizing the microbial parameters on polluted sites. Integrating microbial and chemical analyses in the characterization of an aged polluted site could provide a more comprehensive site pre-evaluation for bioremediation. Prospects of using spatial patterns of specific bacterial taxa as proxies in monitoring polluted sites should be further explored. It is highly relevant to target specific catabolic genes along with the phylogenetic markers in order to gain a detailed understanding of microbial community dynamics in polluted ecosystems. It is noteworthy that

the level of resolution achieved in profiling of structural and catabolic microbial communities in this study could be achieved mostly due to the advancements in next generation sequencing technology.

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